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HAMILTON B. G. ROBINSON

OCTOBER, 1948

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THE EDITOR'S VIEWPOINT

Dawson and Blagg (p. 547) have investigated the effect of human saliva on cholera vibrio. They found that saliva from fifteen healthy young men inhibited growth and lysed cholera vibrio in vitro but that saliva from six cholera patients did not show this degree of antibacterial activity. Here again is evidence of the ability of the saliva to screen out pathogens as they pass through the mouth. The full importance of the saliva in preventing air-borne and food-borne diseases has never been recognized. This local autarcetic immunity may control many invaders when it is operating.

Hunt and Hoppert (p. 553), feeling that their studies have "demonstrated conclusively that there is an inheritance factor for dental caries in rats," suspected that absence of molars in the upper jaw delayed the process of rat caries in the lower molars. As a result of the present study they conclude that occlusion facilitates the development of rat caries in the lower molar teeth of susceptible rats. They further present evidence that mechanical breakage of the upper molars of resistant animals retards development of cavities in corresponding lower molars. Might not the resistance to rat caries in the Hunt and Hoppert strains be the result of inherited resistance to fracture, rather than resistance to decalcification or proteolysis? Is initiation of rat caries, which begins on a fracture, comparable to initiation of human caries which can begin on an intact surface? The implications of this study are far reaching.

Cox, Dodds, Levin, and Hodge (p. 562) give the results of some interesting studies on rat caries. The litter "omitted from consideration" because of a peculiar excavation of the jawbone about the teeth and which formed cavities distinctly different from those formed on corn meal intrigues us. What would bacterial studies of these cavities have shown? Were the cavities more like human caries because a region for plaque formation was afforded? The authors present considerable data to show that fermentable sugars do not promote the initiation of caries in the rat but do accelerate the process once it has been initiated. It has always appeared to us that caries is never initiated on *intact* teeth of rats but only in fracture lesions, with the possible exception of litters such as that omitted in the present study. The relationship between rat caries and human caries therefore must be considered with great caution.

Strålfors (p. 576) compared pH in the dental plaque with lactobacillus counts of 110 individuals. Determinations were made by the antimony electrode method, directly in the mouth. Statistical analysis showed that there were fewer lactobacilli per c.c. of saliva in individuals with higher minimum pH values, although no correlationship between lactobacilli and pH minimum was found below pH 5.0. This is a phase of plaque pH study that is valuable. The study suggested a need for study of the buffer capacity of the plaque.

Strålfors (p. 587), recognizing that paucity of work on buffer capacity of the plaque, as differentiated from those studies on saliva, undertook an investigation. The buffering capacity was found greater in plaque than in saliva and similar in plaque and sediment from centrifuged saliva. The oral bacteria appear capable of overcoming buffering activity and lower the pH to 4.5-5.0. Once this level is reached, it is more difficult for the saliva to neutralize due to the great buffering capacity of the plaque. This explains the rapid drop and slow rise in pH of the plaque in situ.

Hess and Smith (p. 593), having noted conflicting results of studies of the relationship between caries and salivary amylase activity, repeated the qualitative method (Turner and Crane) and devised a reducing sugar method. They found no significant difference in salivary amylase activity between twenty caries-free individuals and thirty-six with cavities. This appears to throw the weight strongly against those who regard the caries-salivary amylase activity as significant, but it would have been more convincing to have weighed salivary amylase activity against caries activity. While salivary lactobacillus counts and similar biologic tests for caries are not in-

fallible, they do give a definite indication of present caries activity while cavity counts are simply historical evidences of disease.

Ennever, Robinson, and Kitchin (p. 599) present a simplified method to study the bacterial plaque which they consider essential for initiation of dental caries in man. A celloidin "tooth" is constructed on a partial denture which may be readily removed for sectioning and study of the plaque. The method may be used to evaluate the efficacy of various drugs against plaque formation and their effect on the microcosm which forms the plaque.

Gorlin (p. 603) investigated the salivary proteins of thirty-five individuals. The mean value was 280.4 mg. per cent of salivary protein with 65.9 mg. per cent deviation in spite of great care to minimize enzymatic action. Variations in salivary constituents, in general, are so great that studies based on small groups of "diseased" and "disease-free" individuals have little significance. Our literature would be enhanced by careful studies of other salivary components.

Sognnaes (p. 609) has reviewed the literature on histologic preparation of enamel specimens and evaluated the common factors of success. Approaching the subject from a different viewpoint than Engels (in press) suggests that extremely long fixation and decalcification is impractical. Sognnaes believes decalcification to be the most critical stage of the process and suggests utilization of a method to suppress CO_2 formation and rapid dialyzing action for minimal "explosive" effect.

Bass and Fullmer (p. 623), continuing a study of the region Bass has termed the zone of disintegrating epithelial attachment cuticle *(ZDEAC)*, question that continuous passive eruption is a physiologic process. This problem arises when ageing changes of the teeth and supporting structures are under consideration. The not too infrequent clinical finding of an old individual with the epithelial attachment at or close to the cemento-enamel junction, brings back the question of change due to trauma (physical, chemical, thermal, or bacterial) vs. physiologic change.

Bird, Kelman, Lerner, Rosenfeld, Totah, and Hodge (p. 629) analyzed the dentin and enamel of teeth from two sides of the same mouth, one side showing normal contour and the other, marked attrition. They found no significant differences in the chemical composition of the teeth from the two sides of the mouth. Unilateral function is often observed, clinically, in instances of periodontal disease and unilateral muscular hypertrophy. This chemical study confirms the impression that unilateral wear may be purely functional rather than being based on differences in tooth composition.

Amler (p. 635) in a study with important clinical inferences, has applied such commonly used drugs as phenol, silver nitrate, fluorine and alcohol, and cavity varnish and cement to dentin in cavities. Using P_{32} as an indicator he found that oxyphosphate cement base was impervious to penetration of P_{32} solution but that all other material investigated increased dentin permeability. Another question mark is thrown at the empiricism of cavity sterilization.

Benzer (p. 640) has shown that physiologic secondary dentin forms without extradental stimulation, that is, in ovarian and unerupted teeth as well as in functioning teeth. The metabolism of the pulp appears to be reduced, with age, as the formation of physiologic dentin slows down, but this does not preclude the formation of pathologic secondary dentin in older teeth under traumatic stimulation.

It has just been announced that Dr. H. Trendley Dean has been appointed as Director of the National Institute of Dental Research and that the National Institute of Dental Research will be in the National Institute of Health. Assistant Surgeon General Bruce D. Forsyth will coordinate the dental research activities with other dental programs in the public health services. In addition to research at the institute's laboratories, at Bethesda, financial grants will be made to outside institutions for research and training, and fellowships and traineeships will be provided in the institute. This culmination of the efforts of many interested groups and individuals to place dental research on a sound foundation is most gratifying. It is hoped that dentistry will accept its responsibilities in this program as well as receive its benefits.

H. B. G. R.

THE EFFECT OF HUMAN SALIVA ON THE CHOLERA VIBRIO IN VITRO:

A Pilot Study*

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INTRODUCTION

THE resistance of tissues of the mouth to infection has long impressed physicians who have suspected an antibacterial action of saliva. However, it was not until Fleming[1] in 1922 first isolated from nasal mucous a bacteriolytic element which was capable of lyzing the test organism, *Micrococcus lysodeikticus*, that scientific evidence of an antibacterial action of saliva was found. Up to 1934, the results obtained were indecisive and there was disagreement regarding the presence or importance of such antibacterial action. During 1934 and in succeeding years other workers,[2, 3] using plating methods, showed conclusively that saliva had an inhibitory action against the diphtheria bacillus. Between 1934 and 1942, Clough[4] and others[5, 6] demonstrated a definite action against other organisms. Fleming and co-workers have reported finding this bacteriolytic agent present in practically all human tissues and secretions except cerebrospinal fluid, normal urine, and sweat. In addition to being especially active against nonpathogenic organisms, it was also found to be in most cases a bacteriolytic or bacteriostatic agent against pathogenic bacteria.

On the premise that the agent present in human secretions and tissues might play a part in body defense against the cholera bacillus, pilot experiments were undertaken.

In the study of cholera, one of the most perplexing questions is the wide variation in susceptibility to cholera of individuals living in comparable circumstances. The *Vibrio comma* being extremely labile in vitro would be expected to be destroyed by the natural defense agents of the body. It is self-evident that not only those who manifest the symptoms of cholera have been infected, but also many others who, for some as yet unexplained reason, exhibit no symptoms of infection, and may or may not reveal cholera organisms upon stool examination.[7] An attempt to explain these individual differences which lead to or resist the disease stimulated the inquiry into the factors which contribute to the self-regulatory defense mechanism of the gastrointestinal tract. Since the consensus is that infecting bacteria gain entrance into the body by the drinking and eating of contaminated foods, the outline of the investigation was to study in vitro, in the order named, saliva, stomach, and intestinal secretions, and when possible, the tissues of these organs. This report is concerned with the salivary activity on cholera vibrio, in vitro.

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*The opinions or assertions expressed in this paper are those of the authors and do not necessarily reflect the opinions or policy of the Navy Department.

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‡Chief Pharmacist's Mate, U.S.N.

EXPERIMENTAL PROCEDURES

Organisms.—Cholera vibrio isolated from a stool specimen of an individual with acute symptoms of cholera infection. The strain was the "Inaba" type isolated by Dr. S. G. Rainsford of our bacteriological department.

Culture Media.—Difco peptone agar, pH 8.5, was used for slants and pour plates. The slants were seeded every twenty-four hours to eliminate the possible transition of cultures into mucoid variances. Tests for the "salivary effect" were made on plates of peptone agar and MacConkey's media.

Saliva.—The saliva used as test samples was collected without stimulation in sterile tubes. A volume of 20 to 30 ml. was considered sufficient. To date, saliva has been collected from twenty-one individuals at various periods during the same day. The procedure was repeated on different days to determine the constancy of salivary secretions. Six cholera patients and fifteen normal, healthy Americans were used as the study groups.

Apparatus.—To test for antibacterial action in addition to colony count by plating methods, a Coleman photoelectric colorimeter and a Beckman spectrophotometer were employed to measure the decrease or increase in turbidity of a prepared standard of cholera suspension.

The conditions of the experiments were varied in the early tests until sufficient data were collected to standardize the procedures. Briefly outlined, the procedure is as follows:

Saliva samples, 20 to 30 ml., are collected in sterile tubes. The sample is centrifuged at 700 r.p.m. for five to ten minutes, and a series of progressive dilutions are prepared from the supernatant liquid with 0.9 sterile saline to make dilutions of 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, and 1:128. One milliliter of a standard cholera suspension, whose optical density is comparable to a No. 10 $BaSO_4$ standard,[8] is added to each saliva dilution. The control tubes are similarly set up but contain no saliva. At zero time, the tubes are read with the aid of a spectrophotometer at a wave length of 350 μ and pour plates made with 0.1 ml. from each sample. After being read and plated, the tubes are incubated at 37° Centigrade. The tubes are read every fifteen minutes and additional pour plates made every two hours. Thus, colony counts were correlated with an increase or decrease in optical density of the cholera suspension.

Experiments were also conducted in an endeavor to determine the nature and properties of the antibacterial agents.

In conjunction with the afore-mentioned procedures, cultures were taken for the purpose of isolating the *V. comma* from the oral cavity, vomitus, and stools of patients showing acute symptoms of the disease. Peptone agar broth was implanted with inoculants from various areas in the mouth and all vomitus and stool specimens were cultured.

RESULTS

The experiments, findings, and conclusions submitted are intended only as a preliminary progress report and are admittedly incomplete.

Under the conditions of the experiments, an antibacterial agent against the cholera vibrio was found in saliva from normal healthy individuals. This agent was present in all samples from the normal, healthy group although the activity demonstrated varied from weak to strong inhibition in the same individual at various times. It was observed that approximately 75 per cent of the antibacterial action of the salivary samples occurred during the first two hours of in vitro contact with the cholera vibrio suspensions (Fig. 1).

The saliva samples from the cholera patients were in the most part found to have only slight antibacterial activity or none (Fig. 2). Saliva having no antibacterial effect appeared to stimulate the growth of the cholera vibrio (Fig. 2).

Evidence that this antibacterial agent may pass through a Seitz filter was shown by the fact that saliva filtrates possessed intensified and similar antibacterial activity. The properties of this agent may be summarized as follows: In addition to killing cholera bacillus, it also kills typhoid bacillus; passes through a Seitz filter; is destroyed at 75° C. in five minutes; is destroyed at 3,000 r.p.m. in thirty minutes; is partially inactivated after twenty-four hour incubation at 37° C.; is partially inactivated after fourteen days' refrigeration at 8° C.; and is completely inactivated by 1/800 N. Acid.

Cultures for cholera vibrio from the oral cavity were found to be only 1.7 per cent positive, and from vomitus, 3.33 per cent positive, in contrast to stool cultures which were 27 per cent positive (Table I).

TABLE I

DISTRIBUTION OF ORAL, VOMITUS, AND STOOL CULTURES FROM FORTY PATIENTS

	STOOLS	VOMITUS	ORAL	THIRD MOLAR OPERCULI	GINGIVAL CREVICES AND POCKETS	EXPOSED ROOT CANALS	TOTAL
Total Cultures	167	33	116	52	58	6	316
Positive Cultures	45	1	2	1	1	0	50

DISCUSSION

The experiments described in the present report demonstrate that there is present in saliva from normal, healthy men an agent which is capable, under the conditions of the experiment, of inhibiting the growth of and lyzing cholera vibrio. However, this agent was found to be either absent or evidencing minimal activity in the saliva from cholera patients.

Fig. 1.—Effect of dilution after two-hour contact and twenty-four hour incubation of saliva from normal healthy person.
1, dilution 1:2; *2*, dilution 1:4; *3*, dilution 1:8; *C*, control.
Isolated opaque colonies are aerial contaminants.

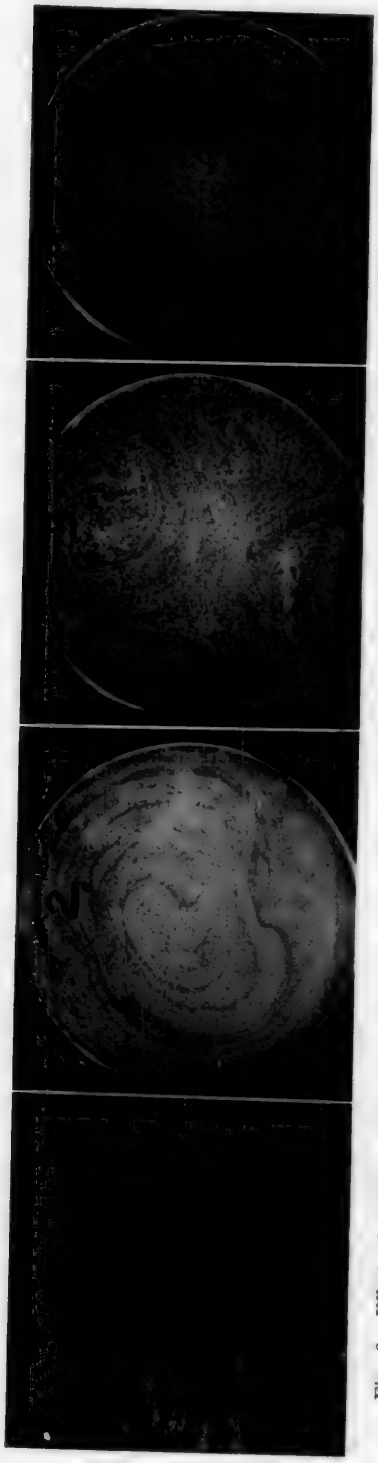

Fig. 2.—Effect of salivary samples on cholerae vibrio. *C*, control; *1*, saliva from normal person, dilution 1:2, showing complete inhibition; *2*, saliva from cholera patient, dilution 1:2, showing slight inhibition; *3*, saliva from cholera patient, dilution 1:2, showing stimulated effect on colony growth.

At present, we are able to offer but little evidence regarding the nature of the antibacterial agent. Attempts made to demonstrate this antibacterial agent in bacteria-free filtrates of cultures of oral bacteria were unsuccessful. This fact plus the demonstration that the agent is present in bacteria-free filtrates of fresh saliva would indicate that it is a product of salivary gland secretion. The inactivation of the agent by heat, refrigeration, centrifugation, and acid offers support to the possibility that the agent has properties similar to an enzyme. It is clear, however, that these preliminary studies justify no final conclusions as to its nature.

In view of the prominence which has been given to claims that cholera vibrio is the cause of cholera, it is interesting to find that saliva is capable of preventing the growth of this organism in vitro. Its role in the mouth in this connection is a matter of conjecture only, but it can only be ignored in considerations of susceptibility to cholera infection when it has been shown that the effects demonstrated in vitro do not operate in human secretions in vivo.

SUMMARY

Saliva from normal healthy men was found to contain antibacterial activity against the cholera vibrio as demonstrated by its ability to inhibit growth and produce lysis of the cholera vibrio in vitro. This activity could not be demonstrated to the same degree in any of the cholera patients studied. The constancy of this agent varied in the same individual at different times. The active principle appeared to be a component of salivary secretion and to have the properties of an enzyme. The inhibitory potency of the saliva appeared to be associated with the state of health of the individual.

The authors wish to express their indebtedness to Lt. (jg) R. H. Weaver (MCR), USNR, for his valuable technical advice, suggestions, and assistance, especially in the use of spectrophotometry for determining the degree of bacterial lysis.

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OCCLUSION AS A FACTOR IN DENTAL CARIES OF ALBINO RATS (RATTUS NORVEGICUS)

H. R. HUNT AND C. A. HOPPERT

From the Departments of Zoology and Chemistry, Michigan State College, East Lansing, Mich.

MAN'S chewing mechanism is very important, because he cannot hastily devour food, as do some of the lower animals, and remain in good health. The causes of dental caries have been sought by nutritionists and bacteriologists, who have created a voluminous literature.

It is becoming increasingly evident that dental caries is the resultant of a number of factors, no one of which is "the cause." The practical importance of this concept is obvious. For example, if an individual has inherited a high degree of resistance to tooth decay, dietary advice and dental hygiene might not be very important within certain limits; whereas if he has inherited a high degree of susceptibility, then periodic inspection of his teeth, regularly brushing them, avoiding some carbohydrates and eating more of certain minerals or vitamins might be exceedingly important.

This paper reports progress on a study begun in 1937 to determine whether heredity is one cause of susceptibility to dental caries in albino rats. Hoppert, Webber, and Canniff[1, 2] devised a diet that would produce caries in the lower molars of these animals. It consisted of coarsely ground hulled rice, whole milk powder, alfalfa leaf meal, and sodium chloride. It is very important to note that the rice in this ration was ground in a precision grinder set so that about 70 per cent of the rice would be retained on a 20-mesh screen (400 holes per square inch). Hunt and Hoppert,[3-5] and Hunt, Hoppert, and Erwin[7] proved that there are marked hereditary differences with respect to susceptibility to dental caries in rats. A susceptible stock and a resistant stock were developed by phenotypic selection, progeny testing of breeding pairs, and brother with sister inbreeding. The experimental animals were transferred to the described ration when they were 35 days old. The lower molar teeth were examined in the living animal approximately every two weeks until one or more carious cavities were positively identified. The number of days from the date on which the ration containing the coarsely ground rice was introduced to the date at which the first cavity was observed (caries time) was the measure of resistance.

Braunschneider, Hunt, and Hoppert[8] discovered that the substitution of rice flour for the coarsely ground rice in the ration delayed the appearance of dental caries in susceptible rats until they were 100 to 150 days old in

The United States Public Health Service has given generous financial support of the parts of this research now in progress. The National Research Council, the American Philosophical Society, and the William John Gies Fund of the American College of Dentists gave previous generous financial aid.

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94 per cent of the animals tested. Susceptible rats were found to be significantly more resistant to dental caries at 100 and 150 days of age than when 35 days old.

SUSCEPTIBLE AND RESISTANT LINES

Table I summarizes the observations on inheritance for all generations of the inbred susceptible and resistant stocks whose records are now complete, or nearly so. At the present time, 5,879 rats have been entered in our records. It should be mentioned that when a resistant animal dies after it has been eating the caries-producing ration for more than thirty-five days, but at the time of the last observation showed no caries in the lower molars, the caries time for that rat is considered as the number of days it had been eating the cariogenic ration at the time of the last fortnightly observation. Usually the caries time for such an animal greatly exceeds thirty-five days, and it is often as much as 400 or 500 days. The "averages of means" are the averages of sibship means. (A sibship consists of all the offspring of one pair of parents.) The mean caries time for each sibship was computed; then the average was obtained of all the sibship means in that generation. This procedure avoided giving undue weight to large sibships.

TABLE I

AVERAGE OF SIBSHIP MEANS*

	SUSCEPTIBLES		RESISTANTS		DIFFERENCES
GENERATIONS	AVERAGES OF MEANS (DAYS)	NUMBERS OF SIBSHIPS USED	AVERAGES OF MEANS (DAYS)	NUMBERS OF SIBSHIPS USED	BETWEEN AVERAGES (DAYS)
2	57	8	116	8	59
3	43	11	142	18	99
4	37	13	168	16	131
5	32	14	186	11	154
6	29	11	248	14	219
7	38	11	245	22	207
8	29	9	221	18	192
9	30	9	345	15	315
10	30	9	310	28	280
11	22	6	392	17	370
12	24	15	316‡	28	292
13	21	14			
14	18	13			
15	13	11			
16	43†	7			
17	32†	14			

*The sibships used comprise five or more rats each. Average of the first generation, 70 days.

†"Fine" rice in the ration.

‡Record incomplete.

The averages of sibship means in the susceptible line have declined, with some irregularities, from fifty-seven days in the second generation to thirteen days for the fifteenth generation. The rise in the sixteenth and seventeenth generations was due to the change in the cariogenic ration, a more finely ground rice being substituted for the coarsely ground rice formerly used. The averages of means for resistants was 116 days at the second generation. It rose to 248 days for the sixth generation, remained at about this level in the seventh and eighth, increased abruptly to 345 days at the ninth generation, and remained

on this plateau during the tenth, eleventh, and twelfth generations. The difference between the two lines is statistically significant.[7] *The data in Table I demonstrate conclusively that there is an inheritance factor for dental caries in rats.*

OCCLUSION AS A FACTOR IN DENTAL CARIES

One of our original objectives was to estimate the number of pairs of genes responsible for the hereditary difference between the susceptible and resistant lines. The two lines must become highly uniform (presumably homozygous for the genes involved) before we would cross them, and then by means of the distributions in the F_2 and back-cross generations make this estimate.

Hunt, Hoppert, and Erwin[7] (Fig. 1) found that the distributions of susceptible and resistant rats formed conspicuously different types of curves. The curves for the susceptible generations approximated the form of the bell-shaped normal curve with maximum caries times around seventy days or less, and with relatively low variability. The curves for the resistant generations reminded one of a creeping earthworm; they were flat in form, overlapping the susceptible curves at one end and extending in the other direction to caries times of 700 days or more.

In spite of careful selection for high resistance when parents were chosen, brother with sister inbreeding to bring about homozygosity, and progeny testing of breeding pairs, the resistant strain has maintained its high variability with exasperating and discouraging persistency. The standard deviation for caries time increased, irregularly, from 65.3 days in the second resistant generation to 165.6 days at the tenth generation, while the coefficient of variation was .56 in the second generation, rose irregularly to .72 at the eighth generation, then declined to .57 at the tenth.* Contrary to the logic of theoretical genetics, variability had not declined, so that we did not have evidence of the homozygosity we seek. Why did such variability persist? Possibly some circumstance was causing part of the rats to appear more resistant than they actually were, genetically, so that we may have been selecting only moderately resistant animals, in some cases, for breeders.

It was decided to examine the molars in the dissected heads of resistant animals, including problem cases, with the binocular microscope to discover reasons for this peculiar phenomenon. One hundred eighty such heads were studied.† The most striking result of this study was the discovery that in many cases *one or more of the molars on the upper jaw had disappeared,* possibly as a result of breakage due to chewing the hard rice particles in the ration. Evidence will now be presented to prove that when an upper molar tooth in the rat is absent, lack of occlusion delays the carious process in the corresponding lower molar.

Table II shows the condition of all six upper molars in these animals and the presence or absence of caries in the corresponding lower molars. Sometimes

*Computed by Frederick O. Marzke.

†Miss Ardath B. Allen dissected and studied fifty-seven heads, Mr. Thomas K. Barber, forty, and H. R. Hunt, eighty-three heads..

the upper molar tooth had disappeared altogether and the mucous membrane partially or completely covered the hole. The classification of an occasional tooth was uncertain, and such have been omitted from the table. Table II records 351 carious lower molars but only thirty-one of these were third molars, and so we may dismiss further extensive consideration of this tooth. It has not been a frequent site of decay in our research, except that cavities are sometimes found where the second and third are in contact. The significance of damaged upper molars will likewise not be considered at length.

TABLE II

CORRELATION BETWEEN THE CONDITION OF UPPER MOLARS OF RESISTANT RATS AND THE PRESENCE OR ABSENCE OF CARIES IN THE CORRESPONDING LOWER MOLARS*

CARIES IN THE OPPOSING LOWER MOLAR	FIRST RIGHT UPPER MOLAR			SECOND RIGHT UPPER MOLAR			THIRD RIGHT UPPER MOLAR		
	INTACT	MISSING	DAMAGED	INTACT	MISSING	DAMAGED	INTACT	MISSING	DAMAGED
Present	70	8	5	79	0	0	14	0	0
Absent	41	46	9	86	12	3	153	8	2

CARIES IN THE OPPOSING LOWER MOLAR	FIRST LEFT UPPER MOLAR			SECOND LEFT UPPER MOLAR			THIRD LEFT UPPER MOLAR		
	INTACT	MISSING	DAMAGED	INTACT	MISSING	DAMAGED	INTACT	MISSING	DAMAGED
Present	71	7	4	74	2	0	16	1	0
Absent	37	51	9	88	11	3	154	6	0

*Combined data of Allen, Barber, and Hunt.

There was a surprising similarity between the right and left sides with respect to the numbers of carious teeth, missing upper molars, and damaged upper molars. There were 137 missing first and second upper molars, of which 112 (81.8 per cent) were first molars. There were likewise more damaged first upper molars than second. Why should the first molar be destroyed more frequently than the second? It seems probable that it is the first one to attack the food, that it accomplishes most of the reduction of food particles, leaving very much less work, as a rule, for the second molar to do.

The main significance of Table II is in the relation between presence or absence of the upper molars and the occurrence of caries in the corresponding lower molars. Only the first and second pairs will be considered. There were 546 intact upper molars; 294 (53,85±1.44 per cent) of the corresponding lower molars showed caries. The total number of missing upper molars was 137, and only seventeen (12.41±1.90 per cent) of the opposing lowers had carious cavities. The difference, 41.44±2.38 per cent, is decidedly significant. Absence of the upper molar is correlated with absence of caries in the lower molar of the pair in rats which were not in an experiment designed to test this relationship critically.

The data in Table II justify the hypothesis that the carious process in a lower molar is encouraged by use, by pressure against the upper molar. But many of these animals were killed weeks after the last examination for cavities. Suppose that a rat first showed a cavity in the lower right first molar when 286 days old. Being fairly resistant, it was saved as a breeder or potential

breeder for four months, when it was killed because it was too old for further breeding. The cavity in the lower right first molar is easily found with the binocular microscope, and it is discovered that the upper right first molar is gone except for a few protruding pieces of the roots When did this upper molar disappear? If it disappeared a considerable time before the rat was 286 days old, it probably contributed little or nothing to the formation of the carious cavity; but if it functioned throughout the formation of the cavity and went to pieces after the cavity appeared, it may well have facilitated cavity formation, though its absence in Table II might seem to support the view that it did not do so.

Of course, a critical test would consist in removing certain upper molars before any caries had an opportunity to appear in lower molars, then watching for caries in lower molars both in those which occluded with uppers and those that could not.

Table III summarizes Hunt's observations on the first and second pairs of molars (those on the right side being combined with corresponding molars on the left) of rats which were killed less than one month after the last observation of the lower molars. In this group there was less opportunity for upper teeth to disappear subsequent to caries formation in the corresponding lowers.

TABLE III

CORRELATION BETWEEN UPPER AND LOWER MOLARS IN RESISTANT RATS KILLED LESS THAN ONE MONTH AFTER THE LAST OBSERVATION OF LOWER MOLARS*

UPPER MOLAR INTACT† CARIES IN OPPOSING LOWER MOLAR		UPPER MOLAR MISSING† CARIES IN OPPOSING LOWER MOLAR	
PRESENT	ABSENT	PRESENT	ABSENT
57	123	5	78

*Selected from Hunt's data in Table II. In all of Hunt's data, if a lower molar had disappeared, it was recorded as having been carious. Obvious fracturing of lower molars was not counted as caries.

†Only the first and second pairs of molars are included.

Intact upper molars were associated with carious lower molars in 31.7±2.3 per cent of the pairs. Where the upper molar was missing, 6.0±1.8 per cent of the lowers were carious. The difference, 25.7±2.9 per cent, is very significant statistically.

Table IV gives the same type of information for resistant rats which were killed at varying intervals after they had been feeding on the caries-producing diet for 300 days or longer. This class included some of our "problem children" which ate the cariogenic diet for hundreds of days and sometimes "died of old age" without caries in lower molars. Here again the frequency of caries in the lower molars when the upper molars were intact (17.5±2.3 per cent) was significantly higher than the incidence of caries when the upper molar was absent (5.8±1.9 per cent). The difference (11.7 per cent) had a probable error of 3.0 per cent. The lower percentages of carious lower molars were probably due to the fact that these rats possessed a high level of hereditary resistance.

These facts certainly support the theory that when an upper molar remains, its contacts with the lower molar encourage caries production, but if the upper

TABLE IV

CORRELATION BETWEEN UPPER AND LOWER MOLARS IN FIFTY-FOUR RESISTANT RATS*

UPPER MOLAR INTACT† CARIES IN OPPOSING LOWER MOLAR		UPPER MOLAR MISSING† CARIES IN OPPOSING LOWER MOLAR	
PRESENT	ABSENT	PRESENT	ABSENT
21	99	4	65

*From Hunt's data in Table II. All the rats had been feeding on the caries-producing diet for 300 days, or longer.

†Only the first and second pairs of molars are included.

molar disintegrates fairly early in the life of the rat, the carious process in the corresponding lower molar is delayed or inhibited. However, for all that is known with certainty, all the missing uppers might have persisted until a short time before the death of the rat, and the lowers failed to develop caries regardless of their presence.

It was decided to carry out a critical experiment meeting the following requirements: (1) one or more of the upper molar teeth on one side only to be removed at an age when this could easily be done but before the carious process had begun; (2) the lower molars on the unoperated side to serve as experimental controls to the lower molars on the operated side; (3) the day of the operation to be definitely known; (4) a genetically homogeneous strain of rats to be used so that the specific effects of minor variations in the operation could be determined; (5) some of the animals to be killed at various stages in the experiment to determine what was happening in the mouth.

This experiment was carried out with 108 rats (forty-eight males and sixty females) from the fifteenth generation of susceptibles. This generation was highly susceptible to dental caries as shown by the average caries time of thirteen days (Table I). Its uniformity in this respect was shown by the fact that the most extreme caries times were five days and twenty-four days.

The etherized rats were operated upon when they were approximately 30 days old. One to three of the upper molars on one side only were cut out with small bone forceps. Contrary to intention, the mucous membrane near the tooth was usually injured, and the resulting bleeding made it difficult to determine how many molars had been destroyed. Judgment was based on the tooth fragments clinging to the forceps and picked out of the mouth. Technic improved as the experiment progressed. The dissected mouths of thirty-seven of the rats were subsequently examined with the binocular microscope. The first twenty-one animals operated upon were sacrificed early. In eight of them, the upper first molar had been removed, and in the remaining thirteen, the first and second were gone. In addition, sixteen rats were killed at the end of the study of their caries development. They had been operated upon later than the twenty-one. One of the sixteen lacked the upper first, thirteen lacked the first and second, and in two rats all three molars were missing on one side.

These operated animals were maintained like the others, placed on the caries-producing diet when 35 days old, and the lower molars on both sides were examined for cavities approximately every two weeks. The twenty-one animals mentioned were killed ninety-two to 103 days after being placed on the

cariogenic diet. All of them had developed cavities in the lower molars on the unoperated side of the mouth. The lower molars on the operated side were studied with the binocular microscope. The thirteen animals which had eaten the caries-inducing food mixture for ninety-two to ninety-three days showed no carious lesions in the lower molars on the operated side. The eight remaining rats had been on the standard diet 102 to 103 days. Two of them showed no cavities in the lowers on the operated side, one had developed a cavity, and in the remaining five the cavities were doubtful or small in the molars opposite the blank spaces in the upper jaw. Apparently a carious lesion is likely to become visible under these circumstances after about one hundred days of feeding on the food mixture containing coarsely ground rice.

Table V summarizes the facts concerning the eighty-seven rats whose caries history was followed through a number of months. This experiment provided unusually conclusive controls, because every operated rat had within itself a control—the unoperated side of the mouth. In addition, the fifteenth generation of susceptibles was a product of long selection (phenotypic and genotypic) and close inbreeding, so that it was strikingly uniform. Carious cavities appeared in the lower molars on the unoperated side in all the rats. In twenty rats cavities had not developed in the lowers on the operated side, so that the caries time for these teeth was taken as the number of days such animals had consumed the cariogenic diet to the date of the last examination of the living rat.

TABLE V

CARIES TIMES FOR LOWER MOLAR TEETH OF FIFTEENTH GENERATION SUSCEPTIBLE RATS AFTER ONE OR MORE UPPER MOLAR TEETH HAD BEEN REMOVED AT ABOUT 30 DAYS OF AGE

RIGHT UPPER MOLARS REMOVED (31 RATS)		LEFT UPPER MOLARS REMOVED (56 RATS)	
AVERAGE CARIES TIME FOR		AVERAGE CARIES TIME FOR	
RIGHT LOWER MOLARS	LEFT LOWER MOLARS	RIGHT LOWER MOLARS	LEFT LOWER MOLARS
135 days	37 days	21 days	166 days

Average for operated side (right + left):	154.1± 6.5 days
Average for unoperated side (right + left):	26.3± .9 days
Difference:	127.8± 6.6 days

Referring again to Table V, the average caries time for the operated side (right plus left) was 154.1±6.5 days, and for the unoperated (right plus left) 26.3±.9 days. The difference between these averages was 127.8±6.6 days. This difference, nearly 20 times the size of its probable error, was very significant in a statistical sense. That is, it was probably not a difference that was due to chance. Had all the rats been observed until every operated side showed a cavity, the difference would have been even greater. But when the experiment had reached the point at which it was clear that the objective had been attained, it was terminated to save money, time and space.

We have, therefore, a decisive demonstration that use of a lower molar in pressing food particles against the upper molar strongly favors the carious process in susceptible rats.

A number of other facts of interest emerged from these operations on susceptibles. The lower left molars on both operated and unoperated sides seemed

to be more resistant than the comparable lower right molars. Only about one-third of the cavities in the fourteenth and fifteenth generation susceptibles were on the left side. Perhaps these facts were related.

Even where the lower molars occluded with their partners on the upper jaw, the average caries time was twice as long (26.3 days) as for unoperated fifteenth generation susceptibles (13 days).

Carious cavities can be formed in lower molar teeth even if the partner on the upper jaw is missing. Sixteen such cavities were seen with the binocular microscope in the experiments just described with susceptibles.

THE MODIFIED CARIES-PRODUCING RATION

We are now proceeding on the supposition that the very coarse, hard rice particles in the food mixture used until recently have been responsible for the mechanical breakage and sometimes the elimination of upper molars in our resistant rats. We now have excellent grounds for believing that such accidents delay the carious process in some of the resistant animals, giving the impression that they are genetically much more resistant than they actually are. If a number of such animals occurred in a sibship, they might raise the mean caries time for that sibship so much as to mislead us as to the genetic worth of that family. This might lead to the perpetuation of some genetically poor stocks which could account for the continuing high variability of the resistant line.

We are now using a modified ration, ingredients and proportions of which are exactly the same as in the previous ration, except that the rice is ground finer by the precision grinder, so that instead of having about 70 per cent of the particles large enough to be retained on a 20-mesh screen, only about 2 per cent are of that grade of coarseness. It is hoped that this will reduce or eliminate mechanical breakage of upper molars. If this troublesome complication can be controlled, we may reasonably expect to create a uniform (homozygous) resistant line which may be crossed with our already uniform susceptibles, and by segregation in F_2 and back-cross generations estimate the number of pairs of genes involved.

Already the revised technic has created difficulties. The new ration will produce caries in both resistant and susceptible lines, but more time is required to do it! Braunschneider, Hunt, and Hoppert[8] discovered that if rice flour is substituted for the coarse rice in the diet, caries production is enormously delayed, perhaps often inhibited, in highly susceptible rats. So if the rice is finer, though not of flour fineness, longer caries times are to be anticipated. This is already evident in the sixteenth and seventeenth susceptible generations whose average caries times have been abruptly increased by the use of the new food mixture.

Another complication that may have to be reckoned with is the age of the rat. Older susceptible rats seem to become somewhat more resistant to dental caries.[8] As the caries time is increased, we may have to devise corrections for the age factor.

Susceptibility to dental caries in rats reminds one of the "extralimited gene substitutions" of Hogben.[9] An extralimited gene is one which produces characteristic and recognizable effects only when the animal is subjected to a specific and somewhat limited kind of environment. The significant environmental variable in this research is the size of rice particles in the ration—something apparently so trivial that one would not, offhand, assign much importance to it. Susceptibility to tooth decay in rats is probably dependent on more than one pair of genes, however.

Dental caries presents a complex problem. The informed will agree that such is the case in man, and we have found that this certainly is true for the rat. One cannot apply to man, uncritically, conclusions true for the rat without confirmatory research on man himself, but research on the lower animals is certainly helpful to medical investigators.

SUMMARY

1. The existence of an inheritance factor for dental caries in albino rats is confirmed.

2. Occlusion facilitates the development of caries in the lower molar teeth of the susceptible strain.

3. There is evidence that the mechanical breakage and final disappearance of the upper molar teeth of resistant animals retards the development of cavities in the corresponding lower molars.

4. More finely ground rice particles are now being used in the diet in the hope that the genotypes of resistant rats may be more accurately evaluated.

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THE EFFECT OF CARBOHYDRATES ON EXPERIMENTAL CARIES IN THE RAT

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THE question of the role of sugar in human caries has been so frequently discussed that no attempt will be made to review the literature herewith.‡

In studies of the relationship of carbohydrates to dental caries in man, it has not been possible to recognize influences operating through oral as distinguished from those operating through systemic nutritional channels. Consequently, the interpretations of the effects of carbohydrates on caries in *man* have been controversial.

A feature of special interest which is gained in studies of the *albino rat* is that, at least to some degree, a separation is possible of the forces initiating caries from those forces advancing already established lesions. The albino rat is unique in that it does not have a significant incidence of dental caries when maintained for long periods of time on diets high in carbohydrates,[1-4] but the rat will show caries-like lesions in a relatively short time if maintained on rations containing coarse particles of maize or rice.[5] The nonoccurrence of caries in carbohydrate-fed albino rats indicates that carbohydrates are not potent initiating factors of caries in this species; the occurrence of carious lesions after diets containing identifiable initiating factors shows the albino rat to be susceptible to a tooth injury which our data show develops further like true dental caries.§

Shibata,[6] in 1928-1929, fed to rats diets containing rice and greens mixed (to the extent of 5 per cent) with sucrose, glucose, lactose, and maltose, respectively. He failed to recognize the importance of mechanical factors and ascribed the carious attack to the added carbohydrates. Subsequent to the demonstration of the role in rat caries of coarse corn particles by Hoppert, Webber, and Canniff[5] in 1931, Rosebury, Karshan, and Foley[7] were able to show that a similar causal role could be ascribed the coarse rice particles in the diets used by Shibata.

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‡The reader may find an introduction to the literature in the "General Analysis," p. 254 of Dental Caries, ed. 2, 1941, Lancaster Press, Inc., Lancaster, Pa.

§For the purpose of this discussion, it is suggested that two stages be clearly distinguished: (1) initiation of caries, and (2) promotion, development, acceleration, and cavitation. It is not necessary to assume that the factors involved in either are mutually exclusive or that these factors are ever present alone.

The recent findings by Keyes[9] that a high sugar diet increases the incidence of caries in *hamsters* has opened up the entire field of the relationship of sugar to experimental caries. There is some additional evidence available from studies on *albino rats*[10, 11, 12] and on *cotton rats*.[13, 14, 15]

It is peculiar in the light of the notable attention which has been directed to this subject that no studies have appeared heretofore of the effects on *rats'* teeth of adding excessive amounts* of sugar to the Hoppert-Webber-Caniff coarse corn particle diet.[5] Several such experiments are described in the following pages. However, the data in this report are not offered as conclusive evidence in a long debate, but only to point out a procedure which may be exploited profitably to gain additional knowledge.

The data reported herewith present, *first*, evidence that the maintenance diets containing no coarse corn, regardless of their starch and sugar content, produce caries only rarely when rats are maintained on them for as long as eleven months. *Second*, when rats are placed on a caries-producing diet for a short period and then subsequently maintained on diets containing various carbohydrates in excessive amounts, it was believed that the stage was set for testing the effect of carbohydrates on the incidence and continuation of rat caries. Three experiments have been performed. In the first one, the HWC† diet was just diluted by adding an equal weight of powdered sugar. In the second one, the HWC diet was followed by various diets containing no coarse corn but with high levels of selected carbohydrates. In the third experiment, each HWC ration was prepared using, as nearly as possible, only one carbohydrate, the same carbohydrate that was present at a high level in the diet during the continuation period when no coarse corn particles were present.

THE NONOCCURRENCE OF CARIES IN RATS ON DIETS WHICH DO NOT CONTAIN COARSE PARTICLES

Albino rats at 28 days of age were distributed into three groups and fed the rations shown in Table I. When the rats were 112 days old, they were mated. Some of the offspring were used in the experiments described in the following pages on the promotion of caries after initiation. One, two, and three litters of young, averaging 8.5 rats per litter, were born on these rations, and over two-thirds of the litters were weaned on each of the high carbohydrate diets. Many of the young were raised to maturity and third and fourth generations were obtained. Thus the rations appear to be reasonably complete for growth and reproduction.

The teeth of all rats which died during the course of experiments or which were killed for various reasons have been examined for caries. With one curious

*Rosebury and Karshan[8] added up to 28 per cent sugar to diets in which coarse rice or corn particles were present with inconclusive results.

†The coarse corn meal ration of Hoppert, Webber, and Canniff.[5]

exception,* no caries were found in any rats that had not been on diets containing corn meal. Two rats of the 76 studied showed small cavities. One rat on the glucose diet (Table I) had a small cavity in an upper molar; another rat on the same diet had several questionable cavities. In the other 74 rats that had not been on the corn meal diets, no caries was found. These rats ranged in

TABLE I

PERCENTAGE COMPOSITION OF THE DIETS USED IN THE STUDY OF THE COMPARATIVE EFFECTS OF DIFFERENT CARBOHYDRATES IN THE RATION OF RATS

	STARCH DIET	SUCROSE DIET	GLUCOSE DIET	LOW CARBOHYDRATE DIET*
Corn starch	65.5	–	–	12.0
Sucrose	–	66.0	–	–
Glucose hydrate	–	–	69.3	–
Hydrogenated fat†	5.0	5.0	4.5	26.0
Butter fat	5.0	5.0	4.5	26.0
Casein‡	20.4	20.0	18.1	30.0
Salt mixture§	4.1	4.0	3.6	6.0

*Note that the low carbohydrate diet is a high fat diet. All diets were designed to be isocaloric.

†Crisco.

‡Labco casein, chosen to minimize the presence of lactose. It has a minimum of fluorine.[17]

§Osborne-Mendel[15]

Daily supplement of 0.5 Gm. unirradiated dried brewer's yeast, 0.5 Gm. alfalfa leaf powder, and 0.1 drop of olive oil. About 1 Gm. of fresh beef liver was fed twice a week.

age from 3 months to 11 months, and they had been maintained after weaning on diets as listed in Table II.

TABLE II

DISTRIBUTION OF RATS IN THE VARIOUS DIETS

DIET	NUMBER OF RATS WITH NO CARIES
Starch	19
Sucrose	9
Glucose	6
Low carbohydrate	19
Starch, 2nd generation	4
Sucrose, 2nd generation	7
Low carbohydrate, 2nd generation	5
Sucrose, 3rd generation	4
Low carbohydrate, 3rd generation	1
Total	74

Of the total of 76 rats, 28 were on either the glucose or sucrose diet; these sugars have been shown to promote the development of caries induced by cracked corn meal. As only two rats on the glucose diet showed any trace of tooth decay, it can be concluded that sugars have little if any effect in starting rat caries. This conclusion is in agreement with the findings of Lilly,[1] that rats kept for a year on high sucrose diets do not develop caries.

*The rats of a single litter had a striking deformation of the jaw bones which consisted of an excavation of the bone surrounding the teeth. In some cases, the bone had disappeared or perhaps had failed to form to a level of about the tips of the roots of the teeth. The teeth of this litter showed many cavities but of a distinctly different appearance from that due to corn meal. Because of the probable connection between the deformity of the jaw bones and the decay of the teeth, and because of the evident familial relationship, this litter is not regarded as representative and is omitted from consideration.

ACCELERATION OF THE PROCESS OF CAVITATION BY CARBOHYDRATE

Experiment No. 1.—Plan: Cavities were initiated by maintaining the rats on the HWC diet for six weeks. The effect of sugar was tested by drastically increasing the dietary content of this substance, namely, half HWC diet plus half confectioner's sugar, and by giving access to this diet, to the amount desired, for about nine weeks.

A group of 48 albino, Wistar-strain rats (29 male, nineteen female) were put on the Hoppert-Webber-Canniff diet[5] at weaning (about 4 weeks of age) and continued on this diet for six weeks. In the belief that at this time some of the teeth were suffering early damage from the coarse corn particles of the diet, a group of 13 rats (7 male, 6 female) were placed on a diet prepared by mixing equal weights of the HWC diet with powdered confectioner's sugar (grade marked "XXXX"). Since no extra vitamin or mineral supplements were supplied, it is not surprising that these rats grew somewhat less rapidly than did the control group. For example, the experimental male rats weighed on the average about 266 grams after 104 days on the sugar-rich diet, whereas the control male rats averaged about 336 grams.

At the time of sacrifice, the sugar-fed rats were in good coat and condition. Autopsies were performed and weights of each of a number of organs were obtained. The average organ weights of the control and experimental rats are given in Table III. The only abnormality observed in the experimental rats was the presence of striking quantities of nearly pure white fat in the omentum, mesentery, and perirenal regions. These milky fat deposits were not examined chemically. The organs of the peritoneal cavity, stripped of their excessively fatty surrounding tissues, appeared normal and were for the most part of normal average weights. The livers of the experimental rats, both male and female, were somewhat heavier than the livers of the control rats.

TABLE III

AVERAGE ORGAN WEIGHTS OF CONTROL AND EXPERIMENTAL RATS

	BRAIN (GM.)	LIVER (GM.)	SPLEEN (GM.)	KIDNEY (GM.)	LUNGS (GM.)	HEART (GM.)	STOMACH (GM.)
			Male				
Experimental group	1.80	9.89	0.70	1.47	1.60	1.01	0.99
Control group	1.66	9.43	0.78	1.78	1.69	1.15	1.09
			Female				
Experimental group	1.72	7.23	0.58	1.20	1.36	0.74	0.89
Control group	1.71	5.99	0.62	1.14	1.29	0.84	0.92

Blood samples taken from the descending aorta at the time of sacrifice were studied (a) for red blood cell counts and (b) for hematocrit values. The average red blood counts were as follows: control, 8.0 million; and sugar-fed, 8.3 million. Both of these averages are within normal limits for our rats. The average hematocrit readings were as follows: control, 45.6 per cent; and sugar-fed, 44.5 per cent. The latter value is somewhat low, but both values are probably within normal limits.

Fig. 1.—(×11). Lower jaw showing slight caries destruction. The size and place of the teeth may be judged from this picture.

Fig. 2.—(×11). Lower jaw. The major portion of the first molar has been lost and considerably more than half of the second molar is missing.

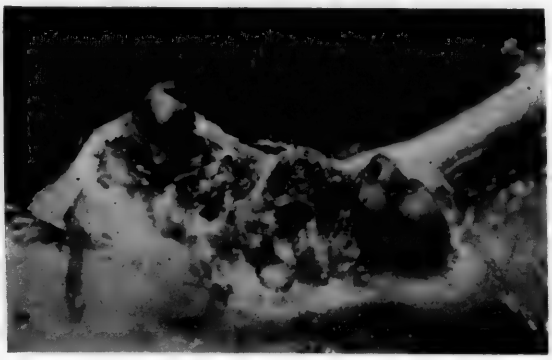

Fig. 3.—(×11). Lower jaw. Advanced destruction. The third molar is intact, and a tiny fraction of the most anterior cusp of the third molar is missing.

Fig. 4.—(×11). Lower jaw. The third molar is practically intact, and the mesial cusp of the first is intact. The rest of the tooth tissue has been broken.

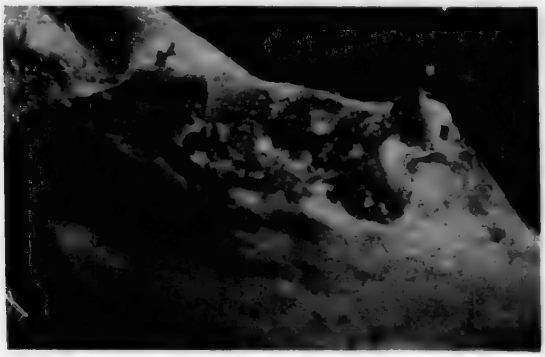

Fig. 5.—(×11). Lower jaw. Only the distal portion of the third molar is present above the gum line. Extremely advanced caries destruction.

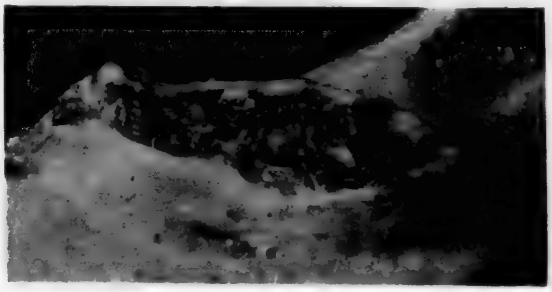

Fig. 6.—(×11). Lower jaw. Practically complete loss of teeth from this jaw. This degree of caries loss was never seen prior to the observation of these rats on high sugar diets.

At death the jaws were freed and the teeth examined under a binocular microscope (15×) according to the technic previously described.[19] No attempt was made to differentiate between missing cusps and carious lesions because of the difficulty in making a clear distinction in the presence of advanced attrition. The data given in Table IV include the average number of teeth having at least one lesion, the average number of teeth totally destroyed, and the average number of cusps missing. In each case the values were calculated per rat.

TABLE IV

DATA ON EXPERIMENTAL CARIES IN RATS FED THE HWC DIET AND IN RATS FED A HWC + SUGAR DIET

DIET	NUMBER OF RATS	AVERAGE NUMBER OF CARIOUS TEETH	AVERAGE NUMBER OF TEETH TOTALLY DESTROYED	AVERAGE NUMBER OF CARIOUS CUSPS
HWC	35	4.9	0.85	12.8
HWC + sugar	13	5.5	1.80	15.0

Comparing the control and experimental groups, there was little difference between the values for the average number of carious teeth (4.9 vs. 5.5) and for the average number of carious cusps (12.8 vs. 15.0) per rat. In sharp contrast to these similarities, there was a 100 per cent difference in the average number of teeth totally destroyed (0.85 vs. 1.80) per rat; this difference was clearly significant by Fisher's "t" test.[20] This finding should be emphasized: twice as many teeth (per rat) were totally destroyed on the diet containing excessive amounts of sugar.

The simplest interpretation to be given these data is that excessive amounts of sugar added to the HWC diet do not initiate experimental caries in the rat; however, existing lesions tend to be extended rapidly and radically. In certain of the jaws of the rats receiving added sugar, not one molar tooth remained. Such a condition had never been observed previously in the scores of rats examined in various experiments in the Rochester laboratory (Figs. 1 to 6).

Summary for Experiment 1.—

1. Forty-eight rats were placed for six weeks on the Hoppert-Webber-Canniff diet, after which thirteen of the rats were given a diet made by adding an equal weight of powdered confectioner's sugar to the HWC diet. These diets were fed for the balance of the experiment of 104 days.

2. Although the experimental rats grew less rapidly than the control rats and laid down excessive amounts of peritoneal fat, there were no gross abnormalities in the organs of either group.

3. On the average, the experimental rats developed the same number of carious teeth and the same number of cusps missing per rat as did the control rats.

4. The experimental rats exhibited twice as many teeth totally destroyed as did the control rats. Apparently, excessive amounts of sugar do not initiate rat caries but existing cavities are rapidly extended.

Experiment No. 2.—Plan: Cavities were initiated by maintaining the rats for eight weeks on HWC diet. Then the colony was divided into groups with various diets as indicated after one week on a cleansing diet. The continuation diets for the next eighteen weeks had excessive amounts of selected carbohydrates.

Rats, at the age of 28 days, were given the HWC diet. Two such diets were used, one containing corn meal as obtained from the market; the other, containing corn meal ground fine, that is, fine enough to be passed through a sixty-mesh sieve. The screen analysis of the original corn meal was: none was retained on a twenty-mesh screen, 84 per cent retained on a forty-mesh screen, 13 per cent retained on a sixty-mesh screen, while 3 per cent passed a sixty-mesh screen. According to Hoppert, Webber, and Canniff,[5] no caries should be expected in the rats given the fine corn meal diet.

The teeth of the rats were prepared for examination by boiling the heads for one-half an hour with a soap solution. The boiled flesh was then easily removed from around the jaw bones. The jaws were removed and flesh brushed away with a tooth brush. The teeth were then cleaned by picking with a fine, sharpened dental broach under a binocular microscope with five power magnification.

After the teeth were dried, an attempt was made to obtain a numerical estimation of the extent of cavities by assigning values to a given size cavity. The values used were: 0 = no visible caries, 1 = a definite cavity, 2 = deep cavity with all cusps intact, 3 = some or all cusps gone, 4 = most of tooth above bone line gone, and 5 = all of crown of tooth gone. It was early found that cavities could not be sharply classified. Therefore intermediate values of ½ were used when desired.

It is obvious that such a numerical scale gives only a rough measure of the degree of caries. Also it is evident that the system does not progress by exact units as "2" is not exactly twice "1", and "4" does not guarantee that the factors have been exactly twice as intense as those which produced caries scored "2." Therefore, averages derived from such numbers must be accepted with caution.

At the end of eight weeks on the corn meal diets, it was found that sixteen of twenty rats on the coarse corn had caries with an average score of 4.8. Contrary to Hoppert, Webber, and Canniff, caries were found in ten of thirteen rats maintained on the fine corn meal diet with an everage score of 2.6.

The entire group of survivors were transferred for one week to a low carbohydrate diet (see Table I). The purpose of this diet was to clean the teeth of the rats of corn meal debris. Two groups of rats were killed at this time and the extent of caries determined (Table V, Initial Phase). These included twenty rats maintained on the coarse ground corn diet and thirteen on the fine ground corn diet. If no cavities were evident, the rats were not considered in the average because continuation of caries was being studied.

The rats were next divided into groups as indicated and allowed free access to the respective diets according to the following schedule: 15 rats were returned to the HWC diet, 12 rats were returned to the fine corn HWC diet, 15 rats were placed on the starch diet (Table I), 16 rats were placed on the sucrose diet (Table I), and 16 rats were placed on the glucose diet (Table I).

The rats were killed after eights weeks on these diets and the teeth examined as described. The results are given in Table V. In the table are given the number of rats, the average caries score, the numbers of rats averaged, and the variability. The last column of Table V gives the summed squares of the deviations from the means, values needed for the interpretation of the means and necessary to show dispersion where the complete data are not given.

TABLE V

AVERAGE CARIES COUNTS OF RATS ON VARIOUS DIETS

DIET	NUMBER OF RATS	AVERAGE CARIES	NUMBER OF RATS AVERAGED	ΣD^2
Initial Phase				
Coarse corn (8 weeks)	20	4.8	19	236.7
Fine corn (8 weeks)	13	2.6	12	84.0
Continuation Diets				
Low carbohydrate	15	3.5	12	118.3
Fine corn	12	4.2	11	65.0
Coarse corn	15	7.5	14	224.3
Starch	15	5.0	13	85.5
Sucrose	16	4.8	9	68.6
Glucose	16	6.5	14	146.3

The interpretation of Table V is that diets high in carbohydrates probably promote rat caries more than does a low carbohydrate diet. Statistical analysis has shown that the chances are about 8 to 1 that glucose promotes rat caries more than does sucrose or starch. Since these odds are not sufficient for a conclusive statement that glucose is more provocative of a continuation of rat caries than is sucrose, it was decided to repeat the study with a more rigidly controlled set of conditions.

Summary for Experiment No. 2.—

1. One hundred twenty-two male and female rats were placed on HWC diets for a period of eight weeks. Twenty rats fed coarse corn and thirteen rats fed fine corn were sacrificed at that time; the average caries scores were 4.8 and 2.6, respectively.

2. The remaining rats were divided into groups of twelve to sixteen each, and they were fed diets containing (a) corn ground coarse or fine, and (b) low or high carbohydrate content, but without corn particles.

3. At the end of an additional eight-week period, the rats maintained on coarse corn had an average caries score of 7.5, whereas those on fine corn had an average score of 4.2. In both these groups, continuation on the diets produced increased caries scores.

4. The rats fed high carbohydrate diets in each instance had higher average caries scores than those maintained on the low carbohydrate diet.

5. When the caries scores of the rats fed continuation diets high in starch, sucrose or glucose, respectively, were compared, inconclusive difference were found.

Experiment No. 3.—Plan: Cavities were initiated in an eight-week period on an HWC diet modified to contain only one sugar. Then the rats were given access to a continuation diet (for eight weeks) high in the same sugar but lacking coarse corn particles.

Rats for this experiment were reared in the Pittsburgh laboratory. In order to provide rats as nearly as possible uniform from each breeding group, litters were reduced to eight after three days and to six after a week. The young were taken from their mothers at 21 days of age and placed on the HWC diet made up with a synthetic milk. The basic composition of this caries-producing diet was as follows: coarse, yellow corn meal, 66 per cent; basic mix, 13.75 per cent; butter fat, washed free of lactose, 8.85 per cent; and selected sugar or starch equivalent to 11.4 per cent sucrose. The basic mix of the above diet was casein, vitamin-free (lactose-free), 4.644 Gm.; Osborne and Mendel salt mixture, 1,257 Gm.; sodium chloride, 0.727 Gm.; alfalfa powder, 2,182 Gm.; and unirradiated brewer's yeast, 1.190 Gm.

The selected sugars were sucrose or glucose. Starch, in an equivalent percentage, was used in the diet of a control group. The casein was chosen to be free of lactose so that the HWC diet contained, as nearly as possible, only a single added carbohydrate. The advantages of this experiment over the preceding one were (1) the diet and life history of the parents were known, (2) the lactation conditions of the litters were practically uniform, and (3) a caries-producing diet was used with a selected carbohydrate component.

The rats were maintained on the caries-producing diets for eight weeks and on the caries-extension diets, the starch, sucrose, and glucose diets (Table I), containing no coarse corn particles, for eight weeks more. The rats were then killed and their teeth examined and scored.

Relative Incidence of Caries Among Males and Females.—In sixty-three cases, a male and a female rat from the same litter ate the same diet. In determining whether the male or female had a larger caries count, all pairs were considered except those in which both counts were zero. If only one had a zero count, that pair was included since this discussion is concerned with incidence and continuation of caries.

In the sixty-three pairs, thirty-nine males exceeded the females in the caries count; twenty-four females exceeded the males. The term exceeded here does not take into account the somewhat dubious averages which made the usual statistical methods applicable only with caution. The data, however, can be treated by the binomial distribution analysis. The mean expected of 63 pairs is 31.5. The distribution found was 24 and 9 or a deviation from the mean of 7.5. The standard deviation, σ, is $63 \times \frac{1}{2} \times \frac{1}{2} = 3.97$. The probability associated

with this deviation is 0.0588 corresponding to chance of 16 to 1. The data, therefore, show that the odds are only 16 to 1 that young male rats are more likely to have caries and more extensive decay than young female rats.

The Progress of Caries.—In order to determine the relative rates of progress of caries on the starch, sucrose, and glucose diets, respectively, the same method given above was used to consider the relative state of decay in pairs from the same litter. However, because of the chance that male rats may have a greater incidence of corn meal caries than females, no pairs consisting of male and female are used in this analysis. This treatment entails loss of data from only a few litters. As progress of caries is being determined, pairs in which either or both show a zero count are omitted from the analysis.

Of the pairs of rats of the same sex and from the same litter but on the starch and sucrose diets, respectively, eight pairs showed more decay on the starch diet and twenty-one on the sucrose diet. The probability associated with this deviation from the expected mean is 0.0155 or the chances are 63 to 1 that caries in young rats progresses faster on a diet high in sucrose than on one high in uncooked corn starch.

Thirty pairs of rats are available on the starch and glucose diets; of these ten show higher caries count on starch and twenty on glucose. The probability of this distribution, if due to chance alone, is 0.0688, or the odds that glucose promotes the caries to a greater extent than uncooked corn starch is 14 to 1.

If the preceding results are combined as starch versus soluble food carbohydrates, then eighteen pairs of rats show the starch-fed animals to have more tooth decay compared with forty-one pairs on the sucrose and glucose diets. The probability of this distribution is only 0.0027, or the chances are 370 to 1 that uncooked corn starch is less provocative of caries than are the soluble food carbohydrates.

Of the pairs of rats fed sucrose and glucose, respectively, twenty-one showed more decay on the sucrose diet and fourteen more on the glucose. The probability in this case is 0.238, or the chances are 3 to 1 that sucrose is more likely to cause continuation of caries than is glucose. Such a probability is, however, well within the limits of a chance distribution.

In Table VI is given the analysis of the decay on the three diets based upon the comparison of specific teeth. For example, in Line 1 of Table VI are given the results of a study of all the pairs of males eating the starch and sucrose diets, respectively. The same teeth in each pair were compared to determine which was more decayed. In a number of pairs the counts were the same for both teeth. In these cases a comparative re-examination of the teeth was made to determine if one was slightly more decayed than the other. Only cases in which decay occurred in both teeth were considered in this analysis.

Of the pairs of teeth in the male rats fed starch and sucrose, respectively, twenty-one showed more decay on starch and twenty-eight more on sucrose. The odds that sucrose in this case is not more provocative of caries are 2 to 1, showing the distribution to be mere chance. In contrast, the chances against sucrose in the case of the female pairs are 100,000 to 1 (Line 4, Table VI).

TABLE VI

ANALYSIS OF THE DATA OF COMPARATIVE DECAY OF INDIVIDUAL TEETH OF PAIRS OF RATS ON STARCH, SUCROSE, AND GLUCOSE

PAIRS	NUMBER OF PAIRS OF TEETH	DECAY GREATEST ON				PROBABILITY	CHANCES
		STARCH	SUCROSE	GLUCOSE	SUCROSE VS. GLUCOSE		
1. Starch vs. sucrose (males)	49	21	28			0.32	2:1
2. Starch vs. glucose (males)	55	27		28			1:1
3. Sucrose vs. glucose (males)	66		37	29		0.32	2:1
4. Starch vs. sucrose (females)	49	9	40			0.00001	100,000:1
5. Starch vs. glucose (females)	38	9		29		0.0014	700:1
6. Sucrose vs. glucose (females)	57		32	25		0.36	2:1
7. Starch vs. sucrose (males and females)	98	30	68			0.0001	10,000:1
8. Starch vs. glucose (males and females)	93	36		57		0.029	35:1
9. Sucrose vs. glucose (males and females)	123		69	54		0.18	5:1
10. Starch vs. sucrose vs. glucose (males and females)	191	66			125	0.00005	50,000:1

A third method of analysis of the data is to determine the average amount of decay per cavity. In all the rats fed the caries-producing and caries-continuation diets containing starch, 174 cavities were produced with a total count of 168. The average count per cavity is thus 0.97. For the rats fed sucrose-containing diets, the data are 206 cavities with a total count of 258 and average of 1.25. The glucose-fed rats had 233 cavities with a count of 250 and average of 1.07. These data on count per cavity are obtainable because of the nature of the values, that is, the caries counts, from which the averages are taken. However, the conclusions substantiate those drawn from the two previously described analyses and are included here as additional evidence.

The data therefore show that sucrose and glucose promote the development of corn meal caries in rats. This experiment does not show whether uncooked corn starch promotes rat caries; that is not essential to the solution of the problem of any caries promoting action of fermentable sugars.

Summary of Experiment 3.—

1. One hundred eighty rats were placed on a specially modified HWC diet in which a synthetic milk replaced the powdered milk usually included. The synthetic milk contained a single sugar, sucrose or glucose; in a control group, uncooked starch was added in an equivalent amount.

2. After eight weeks on these diets, the rats were given new diets in which no corn meal was present, but in which large amounts (about 65 per cent) of each diet was the same sugar or starch which had been in the initial diets.

3. After eight additional weeks on these continuation diets, on the basis of either whole mouth or tooth-by-tooth comparison, it was shown that rats fed uncooked corn starch had less progress of cavitation than rats fed fermentable sugars.

DISCUSSION

The question may be posed: why does sugar provoke the rapid and profound advance of caries in the rat molar? In answer, a few possible factors may be suggested: (a) the solvent action of sucrose for calcium compounds is well established;[21] (b) the sugars may provide a favorable medium for the growth or oral bacteria which are inimical;[22] and (c) sugar may be metabolized by organisms in the mouth to produce sufficient local acidity to decalcify tooth-hard tissues.[23] Experimental rat caries may well prove to be a testing ground for some of the hypotheses which attempt to describe the mechanisms of dental caries.

It seems reasonable to assume that diet may exert an influence on tooth health in ways which may be divided into three phases: (1) dietary factors in the development and calcification of teeth (pre-eruptive); (2) dietary factors in the erupted teeth but prior to the appearance of carious lesions (initiating factors); and (3) dietary factors in the progress of caries (cavitation).[10]

1. Pre-eruptive Factors. No attempt has been made in these experiments to control the pre-eruptive factors. In the third experiment, the maternal diets were considered to be part of the experiment. However, the only importance to the question as to the effects of starch versus fermantable sugars in the diets of the offspring is the clearly established fact that the young rats were susceptible to caries.

2. Initiating Factors. The initating factors were not studied in these experiments; all rats were placed on HWC diets in each of the three tests. A review of the literature indicates that the fermentable carbohydrates would not initiate caries except through a possible coarse particle effect. Thus, McClure[12] found a significant incidence of caries in rats fed granulated sugar as compared with rats fed finely ground sugar. In the experiments reported herewith, coarse corn particles were used as the initiating agents in all cases. In the third test, in order to control the influence of the carbohydrate as far as possible, the same sugar was added to the HWC diets as was used in the continuation diets. The effect of this procedure was only to add the specific promoting effect (if any) of the carbohydrate during the initiating period.

3. Extension Factors. Only one of the many possible factors involved in the destruction of tooth tissue has been studied, that is, the importance of the fermentable carbohydrates. The fermentable carbohydrates, sucrose and glucose, permit more rapid extension of existing cavities than does uncooked corn starch. Whether the initiating agents in experimental rat caries and in human caries are comparable, and whether the mechanisms for the enlargement of cavities in rat molars and in human molars are similar, are problems beyond the scope of this experiment. However, if an application of these findings to human caries prevention can be made, it would seem to stress the importance of insuring the perfection of tooth structure, because once the caries process is initiated, modern civilized diets would accelerate it.

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STUDIES OF THE MICROBIOLOGY OF CARIES

II. The Acid Fermentation in the Dental Plaques in Situ Compared With Lactobacillus Count*

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INTRODUCTION

ACCORDING to the chemico-parasitic theory advanced by W. D. Miller in 1890,[1] the decalcification of the tooth substance is caused by acids produced from carbohydrates by bacteria growing in the mouth. It has been shown by several investigators (Holt, La Mer, and Chown,[2] Enright, Friesell, and Trescher,[3] Karshan and Rosebury,[4] Bibby, Volker, and Van Kesteren[5]) that decalcification of tooth enamel probably occurs at a pH of about 5.0 and below under mouth conditions. The normal pH range of the saliva is from 6.0 to 7.5, although even lower values, to 5.3, have been reported by some authors; consequently, the enamel cannot be dissolved by the saliva. It is a well-known clinical fact that dental caries occurs only at certain locations on the teeth: at the retention places, that is, the fissures, the interproximal areas, and the gingival region of the labial surfaces. Here, caries is established under an adherent film rich in bacteria, the so-called plaque first demonstrated by Williams.[6] Our knowledge about the composition of the dental plaque is very scanty since it is little investigated, but it is generally supposed to contain precipitated saliva proteins, leucocytes, epithelial cells and other substances, and a great mass of bacteria. It seems to be of very great interest to investigate the changes in hydrogen-ion concentration during fermentations in these plaques.

PREVIOUS WORK

Hanke[7] used methyl red as indicator and showed that superficially dried carious enamel or dentin had an acidity of pH 5.0 or below. He also demonstrated that plaques become acid fifteen minutes after ingestion of cane sugar.

Stephan[8] used a microcolorimetric test. The plaques were removed from the mouth and the pH determined under the microscope with the aid of indicators. The pH of plaques varied from 4.6 to 7.0. The average was 5.9. The pH of debris from carious cavities varied from 4.2 to 6.2 (average, 5.2), that is, 0.7 pH unit below that of the plaques.

Later, in 1940 Stephan[9] determined the pH of buccal plaques and carious lesions directly in the mouth with the antimony electrode. It was shown that rinsing the mouth with a solution of glucose, sucrose, or starch caused a rapid fall in pH, but that lactose caused a relatively slow pH drop.

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Miller, Muntz, and Bradel[10] scraped off the plaque material and suspended it in 0.45 c.c. distilled water. Initial pH and lactic acid content were determined; glucose was added, and after thirty minutes of incubation at 37° C., pH and lactic acid content were determined again. The drop in pH and increase in lactic acid were calculated. The quantity of produced lactic acid varied with the amount of plaque material. No difference was found between caries-active and caries-immune individuals.

Fosdick, Campaigne, and Fancher[11] and Fosdick and Burrill[12] removed the plaque material from the teeth after the ingestion of carbohydrates and determined the pH with a microglass electrode. There was a drop in pH of material removed from the teeth of caries-susceptible individuals but not from the caries-immune.

Stephan[13] investigated sixty-five individuals. By clinical and roentgenological examinations the subjects were divided into five groups of different caries activity. The extent and duration of the pH decrease varied with the caries activity, cases with the greatest caries activity producing the lowest pH. By comparison of the maxillary teeth with the mandibular, the greatest pH falls were on the maxillary teeth.

DISCUSSION OF PREVIOUS WORK

The previous investigations can be divided into three groups:

1. The pH of the plaque was determined by colorimetric method (Hanke,[7] Stephan[8]). The plaque is rich in proteins and nontransparent; consequently, the colorimetric method must be considered unreliable.

2. The pH of the plaque was determined electrometrically with a microglass electrode outside the mouth (Miller, Muntz, and Bradel;[10] Fosdick, Campaigne, and Fancher;[11] Fosdick and Burrill[12]). This method is very accurate and reliable. However, in this way, the influence of saliva is eliminated, and only the acid production of the plaque is studied.

3. The pH of the plaque is determined electrometrically with an antimony electrode directly in the mouth (Stephan[9, 13]). With this method the changes in pH are followed in the natural environment with the influence of the saliva and the calcium salts of the enamel surface. One must consider the occurrence of a potential between the plaque and the saliva. The best method should be to use a glass electrode directly in the plaque, but such an electrode must have a very thin glass membrane and consequently is very fragile.

PURPOSE OF INVESTIGATION

The purpose of this investigation was to compare the fermentative properties of the dental plaque with the actual caries activity. As mentioned previously, Stephan[13] in 1944 performed an investigation of this kind using clinical and roentgenological examination to determine the caries activity. However, as it seems very probable that the actual caries activity varies even during short spaces of time, it seemed to be of great value to use the lactobacillus count as

an indicator of the caries activity. The fact that the number of lactobacilli parallels the caries activity has been shown by many investigators (Bunting and associates,[14] Jay and associates,[15] Dean and co-workers,[16] Hemmens and co-workers,[17] and Strålfors[18]).

MATERIAL AND METHOD

The investigation was performed on adult patients in the author's practice and on children at the school dental clinics in Stockholm. Individuals with high caries activity as well as with moderate activity and those completely caries-free were investigated. Altogether one hundred and ten patients were studied.

The patients were instructed not to brush their teeth for three days before the examination and not to eat for four hours before. The pH determinations were made directly in the mouth with an antimony electrode and an electrometric pH meter (Beckman). The antimony electrode was constructed of pure antimony (C. P. Baker's Analysed); a wire was soldered to it, and it was cemented in a glass tube (Fig. 1). The reference electrode was a calomel half cell connected with the floor of the mouth by a rubber tube filled with saturated KCl solution. In the circuit was put a battery with a potentiometer adjusted in such a way that pH might be read directly on the pH meter. Before every examination the pH meter was adjusted against two buffer solutions at pH 7.00 and 4.00 (Beckman).

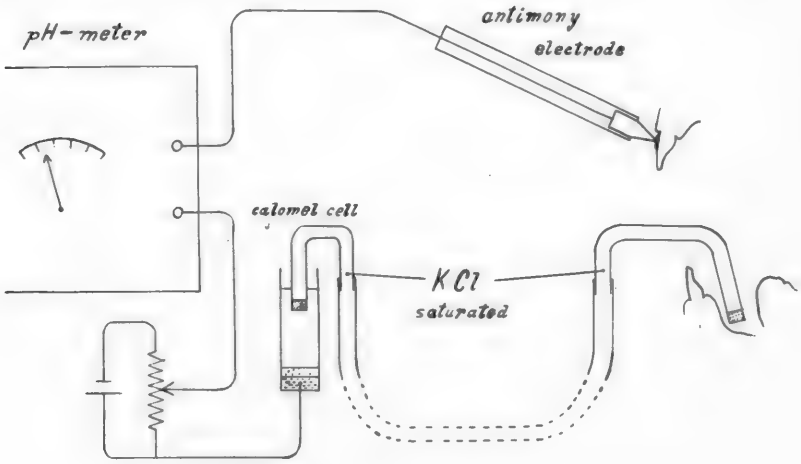

Fig. 1.—Arrangement for pH determinations with antimony electrode.

First, pH was determined in buccal plaque on a maxillary tooth and on a mandibular tooth. Then the patient rinsed the mouth with 10 per cent glucose solution. Again pH in the plaques was determined two, five, ten, fifteen, twenty, twenty-five, thirty, forty, fifty, and sixty minutes after the beginning of the glucose rinse. After each separate pH determination, the antimony elec-

trode was washed with distilled water and dried with filter paper. Immediately after the series of pH determinations was finished, the patient chewed paraffin and spat the saliva in a sterile Petri dish. Cultivation and calculation of the lactobacilli in the saliva was performed in the ordinary way on acid tomato agar plates (pH 5.0).

CORRECTION FOR THE POTENTIAL BETWEEN THE SALIVA AND THE PLAQUE

As the ions, K and Cl, in the saturated KCl solution have almost the same electrolytic mobility, the potential KCl-saliva may be regarded as zero for practical purposes (Fig. 1). But the potential between the saliva and the plaque must not be overlooked. I determined this potential using two calomel electrodes coupled against each other. Each calomel electrode was connected with a rubber tube containing saturated KCl solution and ending with glass tubes drawn out in fine points. The glass tubes were put in KCl solution and the pH meter was adjusted to zero. Then the one was placed at the bottom of the mouth, and the other, in the plaque (Fig. 2). Table I shows the results of typical determinations. The plaque had a potential positive to the saliva. This means that a pH value obtained with the calomel cell connected with the floor of the mouth gives a pH value which is approximately 0.1 pH unit too low. This was also controlled by both the antimony electrode and the point of the glass tube with KCl solution being placed in the plaque (Fig. 3), the position of the glass tube being immediately changed to the bottom of the mouth. In the last case, a pH value 0.1 unit lower than the first case was observed. Because of this potential between saliva and plaque, I have corrected all pH values obtained in the usual way with the calomel cell connected to the mouth floor by addition of 0.1 pH unit.

TABLE I

CASE N:R	POTENTIAL PLAQUE SALIVA IN MV
1	+ 8
2	+ 5
3	+ 10
4	+ 4
5	+ 5
Average	+ 6.4

FINDINGS

Typical pH curves after glucose rinse are shown in Fig. 4. They agree principally with the results reported by Stephan.[13] There is a rapid decrease in pH in the first ten to fifteen minutes, a pH minimum is reached, and then there is a slow pH increase to approximately the original value.

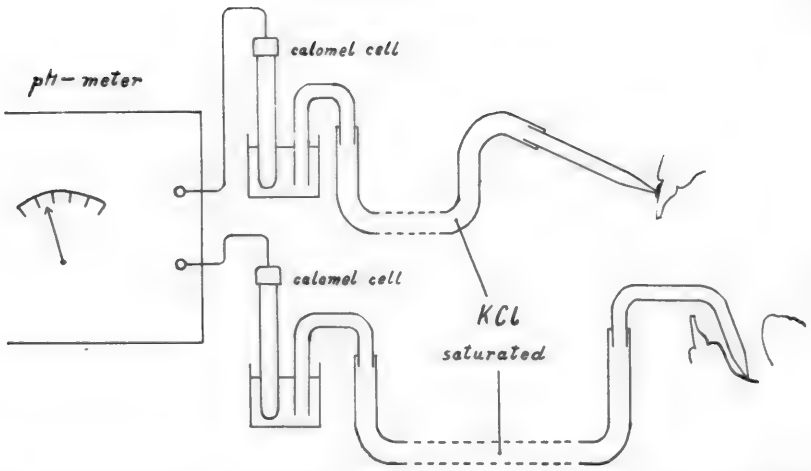

Fig. 2.—Arrangement for determination of potential between saliva and the plaque. Two calomel electrodes are coupled against each other.

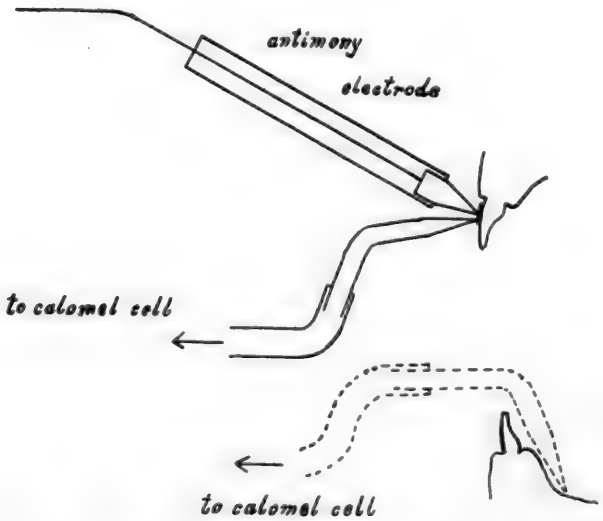

Fig. 3.—Arrangement for comparison of the pH values obtained by connecting calomel cell either with plaque or with saliva.

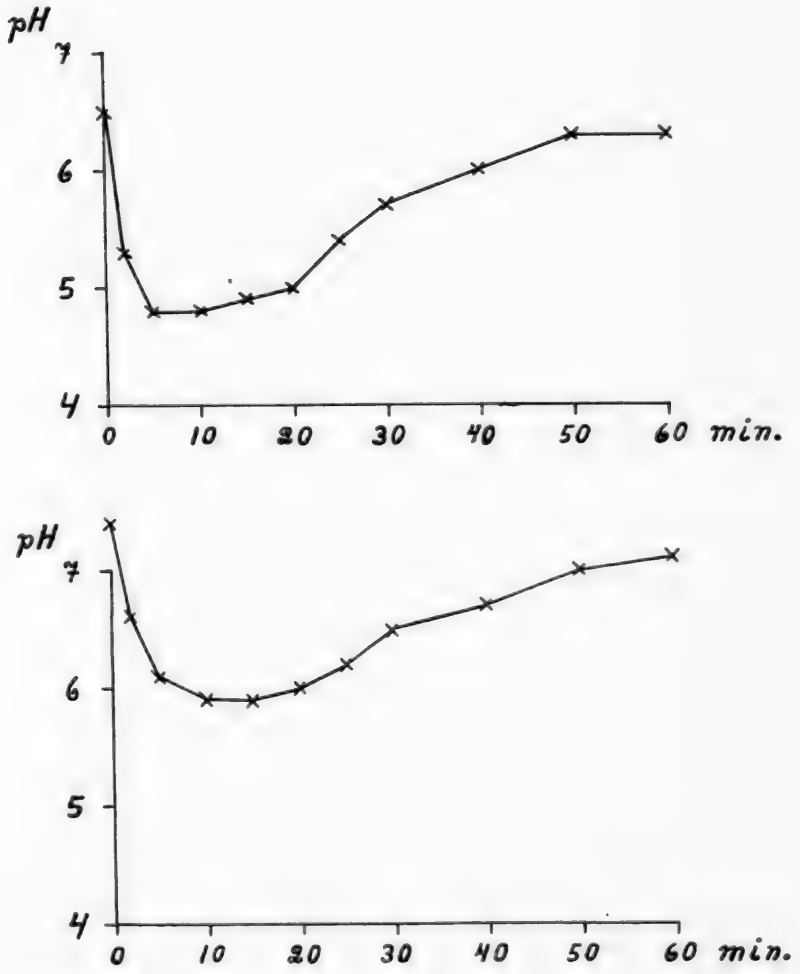

Fig. 4.—Typical pH curves in plaque after glucose rinse. pH minimum 4.8 and 5.9 respectively.

In this way 110 individuals were investigated for pH minimum and for the amount of lactobacilli. Tables II and III and Fig. 5 show the results. It appears that there is clear correlation between pH minimum and the number of lactobacilli.

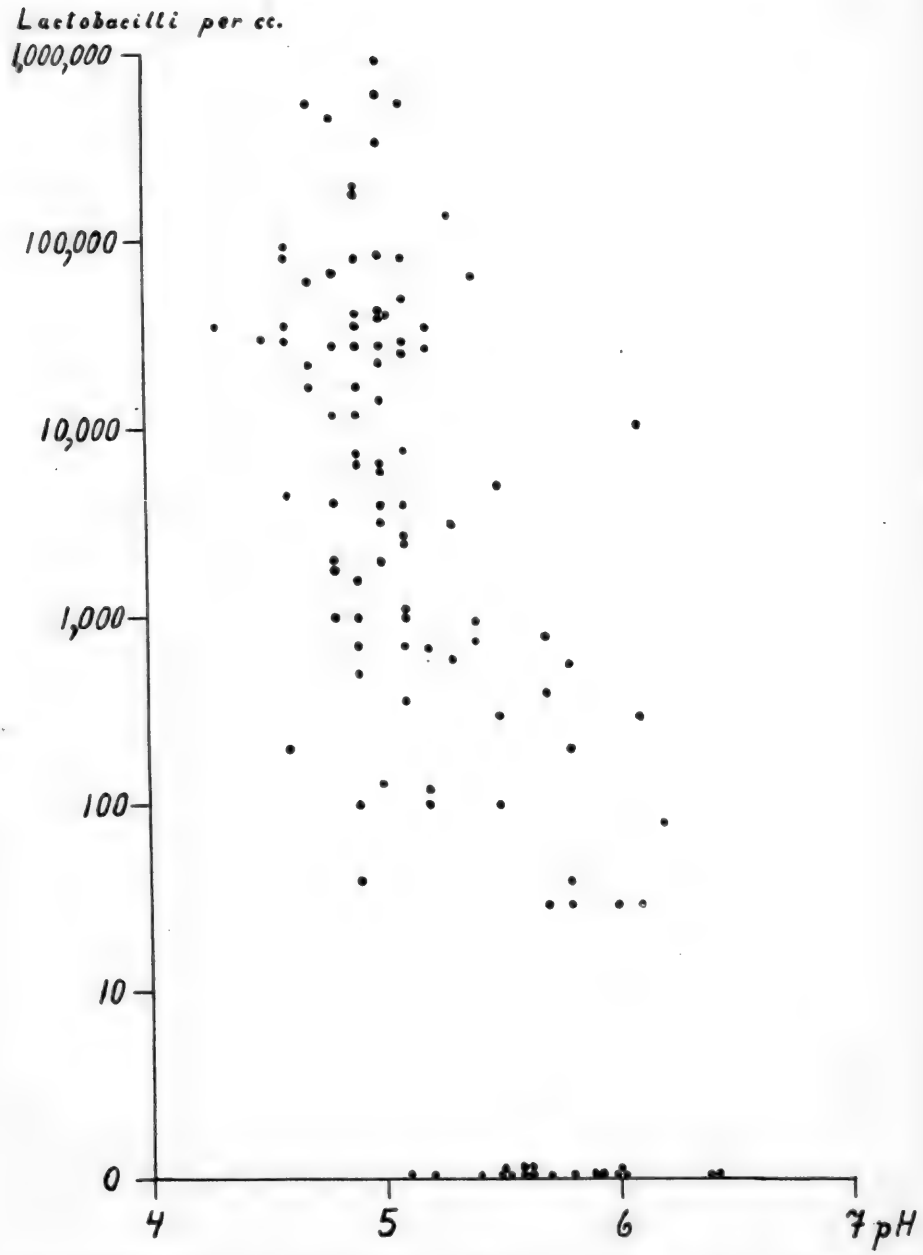

Fig. 5.—Results from investigation of 110 individuals in respect to amount of lactobacilli in saliva and minimum of pH curve in plaque after glucose rinse.

TABLE II

pH MINIMUM	LACTOBACILLI PER C.C. SALIVA	pH MINIMUM	LACTOBACILLI PER C.C. SALIVA
4.3	35,100	5.1	29,600
4.5	31,000	5.1	0
4.6	30,500	5.1	50,500
4.6	36,000	5.1	550,000
4.6	4,500	5.1	81,000
4.6	82,000	5.1	360
4.6	200	5.1	2,700
4.6	95,000	5.1	1,000
4.7	62,000	5.1	2,600
4.7	16,500	5.1	1,000
4.7	22,000	5.1	4,000
4.7	550,000	5.2	120
4.8	28,000	5.2	35,500
4.8	1,800	5.2	100
4.8	1,000	5.2	0
4.8	4,100	5.2	27,750
4.8	11,900	5.2	670
4.8	460,000	5.3	600
4.8	2,000	5.3	137,000
4.8	69,000	5.3	3,200
4.9	178,000	5.4	65,000
4.9	180,000	5.4	900
4.9	17,000	5.4	0
4.9	1,600	5.4	750
4.9	100	5.5	100
4.9	500	5.5	5,000
4.9	6,500	5.5	300
4.9	7,500	5.5	0
4.9	82,000	5.5	0
4.9	700	5.5	0
4.9	1,000	5.6	0
4.9	28,500	5.6	0
4.9	36,000	5.6	0
4.9	41,000	5.6	0
4.9	40	5.7	0
4.9	12,000	5.7	400
5.0	4,000	5.7	800
5.0	3,200	5.7	30
5.0	14,300	5.8	200
5.0	44,000	5.8	0
5.0	620,000	5.8	560
5.0	130	5.8	40
5.0	42,500	5.8	30
5.0	6,000	5.9	0
5.0	340,000	5.9	0
5.0	6,000	6.0	0
5.0	925,000	6.0	0
5.0	2,000	6.0	0
5.0	28,500	6.0	30
5.0	43,000	6.1	10,500
5.0	85,000	6.1	300
5.0	23,000	6.1	30
5.1	7,800	6.2	80
5.1	27,000	6.4	0
5.1	700	6.4	0

TABLE III

pH MINIMUM	NUMBER OF INDIVIDUALS	AVERAGE IN THOUSANDS	HIGHEST VALUE	LOWEST VALUE
4.3	1	35	35,100	35,100
4.5	1	31	31,000	31,000
4.6	6	41	95,000	200
4.7	4	163	550,000	16,500
4.8	8	72	460,000	1,000
4.9	16	37	180,000	40
5.0	16	137	925,000	130
5.1	14	54	550,000	0
5.2	6	11	35,500	0
5.3	3	47	137,000	600
5.4	4	17	65,000	0
5.5	6	1	5,000	0
5.6	4	0	0	0
5.7	4	0	800	0
5.8	5	0	560	0
5.9	2	0	0	0
6.0	4	0	30	0
6.1	3	4	10,500	30
6.2	1	0	80	80
6.4	2	0	0	0

STATISTICAL ANALYSIS

After the pH minimum the material is divided into two groups, those with pH minimum $\leqslant 5.0$ and those with pH minimum > 5.0. Each group is divided into those who have lactobacilli $< 5{,}000$ per cubic centimeter and those $\geqslant 5{,}000$ per cubic centimeter (Table IV).

TABLE IV

LACTOBACILLI	pH MINIMUM $\leqslant 5.0$	pH MINIMUM > 5.0	Σ
< 5000	16	46	62
$\geqslant 5000$	36	12	48
Σ	52	58	110

We calculate χ^2 and obtain $P < 0.01$. This value is quite significant and shows that the number of lactobacilli is *not* independent of the pH minimum. From Table IV we see that of those individuals who have pH minimum $\leqslant 5.0$, $36/52 = 69$ per cent have $\geqslant 5{,}000$ lactobacilli but the corresponding figure for the other individuals is only $12/58 = 21$ per cent.

If we use the figure 1,000 lactobacilli instead of 5,000 we obtain the results shown in Table V.

TABLE V

LACTOBACILLI	pH MINIMUM $\leqslant 5.0$	pH MINIMUM > 5.0	Σ
< 1000	6	40	46
$\geqslant 1000$	46	18	64
Σ	52	58	110

With the χ^2 method we obtain a still lower P value. Table IV, however, seems to be preferred because the figure 6 in one square of Table V is rather little for the calculation of P from the χ^2 value.

The cases with pH minimum $\leqslant$ 5.0 can be divided into those with pH $\leqslant$ 4.8 and those with pH = 4.9 and 5.0 (Table VI).

TABLE VI

LACTOBACILLI	pH MINIMUM		Σ
	$\leqslant$ 4.8	4.9-5.0	
$<$ 5000	6	10	16
$>$ 5000	14	22	36
Σ	20	32	52

The χ^2 method gives a very large P value, P $>$ 0.9, and consequently the number of lactobacilli is not dependent on the pH minimum in these cases with pH $\leqslant$ 5.0.

If we divide those individuals with pH $>$ 5.0 into two approximately equal groups, and then divide these after the number of lactobacilli, we obtain the results shown in Table VII.

TABLE VII

LACTOBACILLI	pH MINIMUM		Σ
	5.1-5.4	$>$ 5.4	
$<$ 1000	11	29	40
$>$ 1000	16	2	18
Σ	27	31	58

Here we cannot use the χ^2 method, as the figure 2 in one square is too little. However, regarding the confidence intervals for the relative figures 11/27 = 41 per cent and 29/31 = 94 per cent, we can judge the difference as significant. This shows that in the group of persons with pH minimum $>$ 5.0, the number of lactobacilli is *not* independent of the pH minimum.

From this analysis we can conclude that the number of lactobacilli depends on the pH minimum in the plaque fermentation in that there are fewer lactobacilli in individuals with higher pH minimum. In those persons with pH minimum $\leqslant$ 5.0, there is no relation between lactobacilli and pH value.

SUMMARY

By means of the antimony electrode and electric pH meter the changes in pH of the dental plaque have been investigated, and at the same time, the lactobacillus count has been made. There is a significant statistical relation between pH minimum and number of lactobacilli. Individuals with higher pH minimum have fewer lactobacilli, that is, lower caries activity than those with lower pH minimum.

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STUDIES OF THE MICROBIOLOGY OF CARIES

III. The Buffer Capacity of the Dental Plaques*

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WHEN the mouth is rinsed with a glucose solution, a rapid production of acid will occur in the plaques. This can be demonstrated by measuring the pH of the plaques with an antimony electrode.[1-3] To ascertain how much acid is produced we must determine the buffer capacity of the dental plaque. I am not aware of any previous investigation in this matter.

METHOD

The patients were instructed not to brush their teeth for four days in order that sufficient plaque material might be collected on the tooth surfaces. A one-drop glass electrode (Beckman) with a cup-shaped glass membrane was adjusted to a buffer solution (pH 7.00) with a Beckman electrometric pH meter (Fig. 1). After the electrode had been dried, it was weighed on an analytical balance (accuracy 0.1 mg.). By air being blown the saliva was driven off the plaques. Then the plaques were scraped off the teeth with a scaler. The plaque material was placed in the cup of the electrode and this weighed again, the difference being the weight of the plaque. One drop of redistilled water was added and the plaque was homogenized in the water with a platinum needle. The pH of the plaque suspension was determined. From a microburette (Linderström-Lang) 0.1 normal HCl was added in portions. The burette permitted the reading of 0.1 c.mm. of the acid. After each portion of acid was added the plaque suspension was thoroughly stirred until the pH reading was constant. In this way, a pH titration curve for the plaque was obtained. For the same individual, a titration curve of the saliva was done as well. In this case, the ordinary bulb glass electrode was used.

FINDINGS

Fig. 2 shows a typical titration curve of a plaque and the corresponding saliva. The pH values are given along the ordinate and the quantity of acid in milliequivalents per liter along the abscissa. It appears that the buffer capacity of the plaque is much greater than that of the saliva, especially below pH 6.0.

In Table I are calculated the buffer values for eight individuals for different pH intervals. In the upper part are the figures for the plaque and the saliva in milliequivalents per liter, and below are the figures for the relation

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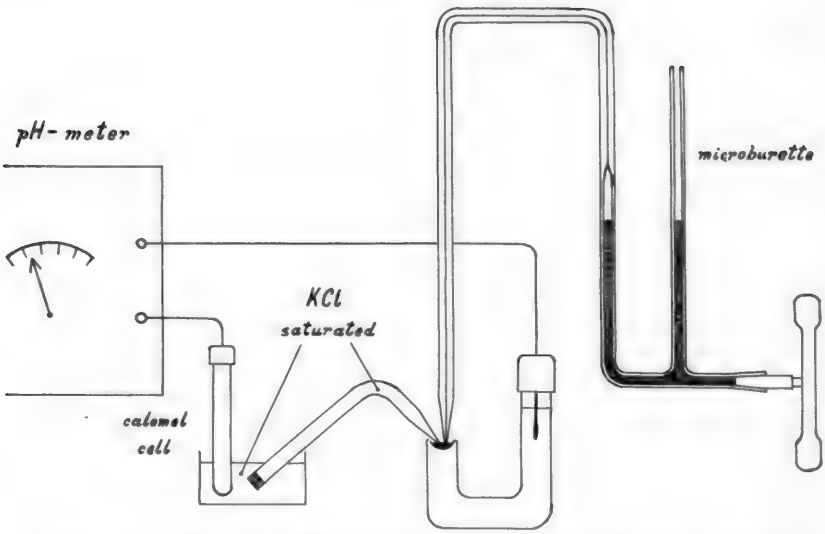

Fig. 1.—Arrangement for determination of buffer capacity of plaque material.

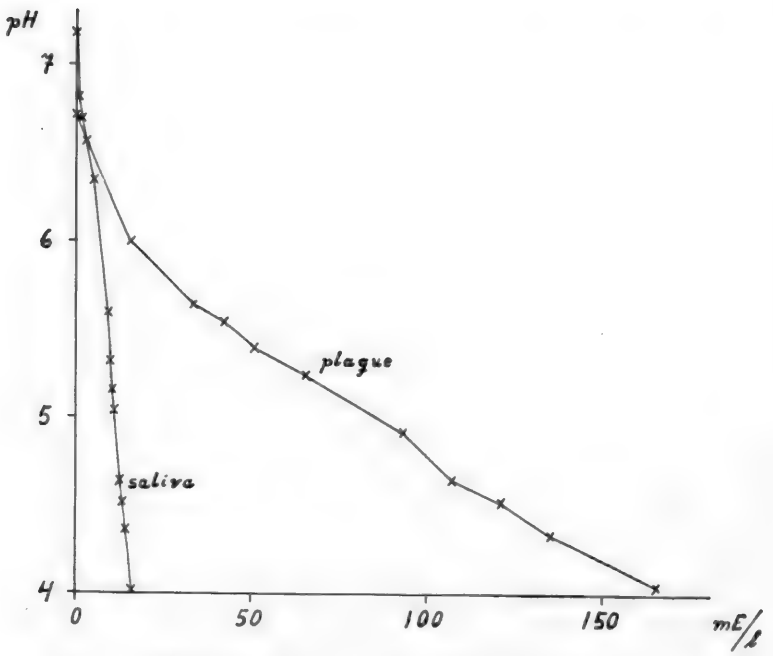

Fig. 2.—Titration curve of a plaque and the corresponding saliva.

TABLE I

TITRATED ACID IN ME/L

PATIENTS	A.S.	P.A.	C.B.	S.L.	Å.L.	B.M.	H.R.	R.T.
				Plaque				
	6.7–6.0	6.65–6.00		6.75–6.00	6.85–6.00	6.40–6.00	6.72–6.00	6.7–6.0
To pH 6.0	14.4	7.39		10.22	16.45	6.09	15.83	12.5
			5.77–5.00					
pH 6.0 to 5.0	28.2	29.23	32.67	19.65	44.75	42.06	74.05	36.5
pH 5.0 to 4.0	81.7	46.02	50.66	62.41	65.2	68.62	77.55	61.0
Totally to pH 4.0	124.3	82.64	83.33	92.28	126.40	116.77	167.43	110.0
				Saliva				
	6.7–6.0	6.65–6.00		6.75–6.00	6.85–6.00	6.40–6.00	6.72–6.00	6.7–6.0
To pH 6.0	3.45	3.15		9.16	3.15	1.72	5.69	6.20
			5.77–5.00					
pH 6.0 to 5.0	4.63	3.35	3.56	7.98	3.25	3.84	4.17	5.12
pH 5.0 to 4.0	6.40	3.94	5.23	7.58	4.73	7.58	5.01	5.91
Totally to pH 4.0	14.48	10.44	8.79	24.72	11.13	13.14	14.87	17.23
β plaque/β saliva to pH 6.0	4.17	2.35		1.12	5.22	3.54	2.78	2.02
β plaque/β saliva pH 6.0 to 5.0	6.09	8.73	9.18	2.46	13.77	10.95	17.76	7.13
β plaque/β saliva pH 5.0 to 4.0	12.76	11.68	9.69	8.23	13.78	9.05	15.48	10.32
β plaque/β saliva totally to pH 4.0	8.58	7.92	9.48	3.73	11.36	8.89	11.26	6.38

of the buffer capacity of the plaque to that of the saliva (β plaque/β saliva). For all the examined individuals the buffer capacity of the plaque is several times greater than that of the corresponding saliva. The difference between plaque and saliva is not so great at the higher pH values as at the lower pH values. For the values above pH 6.0 in the plaque, the original pH value of the plaque is given above the figure for buffer capacity. The original pH value in the saliva was not the same as in the plaque but, as a rule, higher. However, in order to make comparison possible, the calculations in the table are performed from the same pH values.

pH Titration of the Sediment From Centrifuged Saliva.—Saliva always contains suspended particles which make it turbid. By means of powerful centrifuging of the saliva (3,500 r.p.m.), a clear liquid and a slimy sediment was obtained. The sediment was placed in the microelectrode which has been described. By the same technic the buffer capacity in milliequivalents per liter was determined. Fig. 3 shows a titration curve for saliva sediment. It is very similar to the curve for plaque titration with regard to the form of the curve and the buffer values.

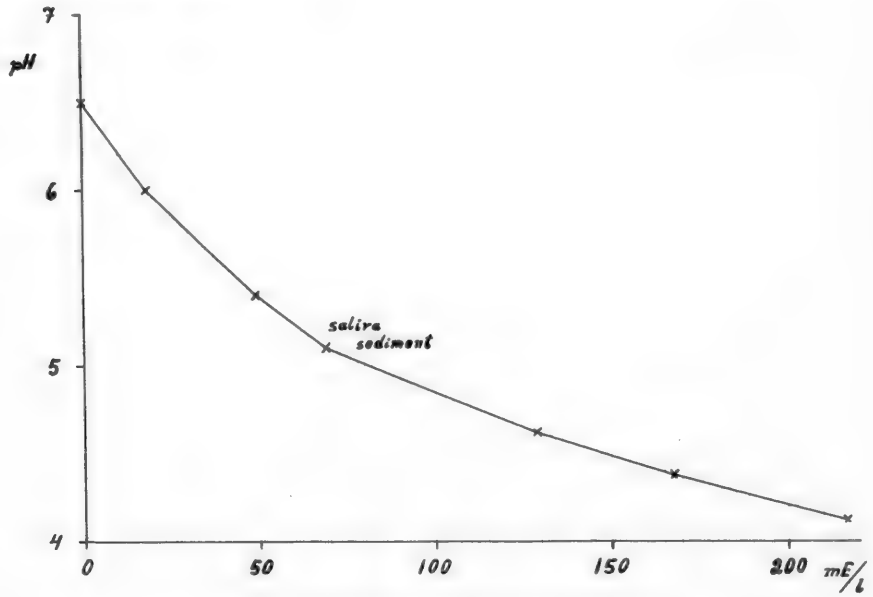

Fig. 3:—Titration curve for saliva sediment.

DISCUSSION

For the most part, the plaque consists of proteins from mixed sources (precipitated from the saliva, the greatest part of which seems to be mucin, epithelial cells, leucocytes, and bacteria). The buffering of the plaque consequently must be considered substantially due to proteins. The sediment from saliva is, of course, not identical with the plaque, but one can suppose that it

is of a very similar composition. There is a great similarity in the buffering ability of the plaque and the saliva sediment in the buffering value as well as in the form of the pH curve.

We know that the acid production by bacterial enzymes is slowed down as pH decreases because the enzymes have their maximum effectivity at a certain pH range, and when pH decreases from this range, the effectivity becomes smaller until finally it is zero. The greater buffering value of the plaque at lower pH values also must be considered to inhibit the pH decrease.

On the other hand, the plaque in the mouth in a few minutes really reaches pH 5.0 and values even as low as pH 4.5. This can be demonstrated with the antimony electrode. This shows that the bacteria are capable of producing sufficient acid to overcome both of the mentioned hindrances. When such a low pH value once is reached in the plaque, it is more difficult for the saliva to neutralize the acid because of the greater buffer capacity of the plaque. The plaque stores the acid and releases it only slowly. This explains the pH curves obtained in the mouth with the antimony electrode. There is a rapid fall in pH but, as a rule, a slower rise (Fig. 4).

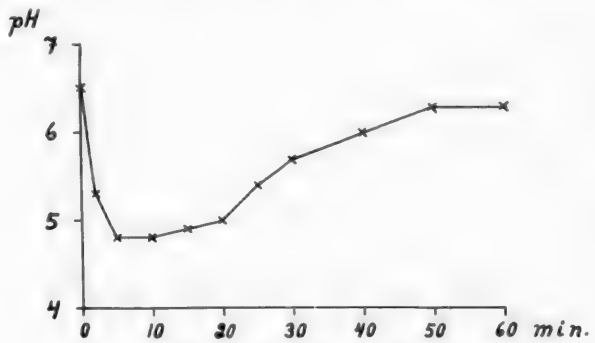

Fig. 4.—pH curve in plaque after glucose rinse.

In experiments to simulate the rapid fermentation in natural dental plaques by using bacterial suspensions with high microbic cell concentration (Stephan,[4] Strålfors[5]), the high buffer capacity of the dental plaques must be considered. The substrate for the fermentation in these model experiments must have the same buffer capacity as dental plaques and not the same as the saliva as supposed by Stephan.[4]

SUMMARY

The buffer capacity of dental plaques has been determined and compared with that of the saliva. It is found that the buffering is greater in the plaque than in the saliva, especially at the lower pH values. The importance of this fact to the understanding of the caries is discussed. It is suggested that the plaque has the ability to store the acid and to hinder the saliva from neutralizing it. It is showed that there is great resemblance between the buffering of the plaque and the sediment from centrifuged saliva.

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THE SALIVARY AMYLASE ACTIVITY OF CARIOUS AND NONCARIOUS INDIVIDUALS

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PICKERILL (1924)[1] found a difference in the degree of alkalinity and the ptyalin index between the salivas of caries free and caries susceptible children. Working with native Maori children, he found that the mouth washings of fifty Maori children who were free from dental caries were six times as alkaline and had a ptyalin activity twice as great as the washings from fifty European children who were susceptible to dental caries. Pickerill employed a semiquantitative method for the determination of ptyalin or salivary amylase activity based upon the visual estimation of the rate of disappearance of the blue color given by iodine and starch.

In 1933 Hubbell[2] found no consistent differences between the amylase activity of the salivas of fifteen children, 9 to 16 years old, who were free from dental caries and a similar group who had active caries. Hubbell employed the picric acid reduction method of Myers and Dellenbaugh[3] for the estimation of amylase activity.

Florestano, Faber, and James[4] in 1941 employed a quantitative spot plate procedure involving the starch-iodine test and reported a series of experiments upon 166 adults. In contrast to the work of Pickerill, they found a marked increase in the activity of salivary amylase in the saliva of carious individuals. Turner and Crane[5] in 1944 confirmed the findings of Florestano, Faber, and James in a series of children, 9 to 16 years old. On the other hand Bergeim and Barnfield,[6] using the qualitative iodine test, were unable to find any correlation between the number of carious lesions and amylase activity.

With these variances in findings in mind, we have undertaken to repeat the qualitative or semiquantitative work of other investigators and to devise a rigorous quantitative method for the determination of the activity of salivary amylase.

EXPERIMENTAL

Qualitative Method of Turner and Crane.—In this method 1 c.c. saliva is mixed with 1 c.c. of 1 per cent starch solution, and four-drop aliquots are tested with iodine solution at short intervals. In contrast to the results of Turner and Crane, we found that all salivas, both from carious and noncarious individuals, gave a yellow color with starch, indicating complete hydrolysis, within one minute. In an effort to reduce the rate of reaction of the salivary amylase, the tests were repeated on a 1:50 dilution of the saliva. While this process increased the time for the development of the yellow color, there was no consistency in the results. Saliva from the same individual taken upon successive days produced a yellow color in from six to thirty-seven minutes. When the procedure was tested upon a series of eighteen noncarious individuals, the time for complete hydrolysis varied from three to thirty-seven minutes. In eight individuals with from five to twenty carious surfaces, the time varied from one to twenty minutes. No correlation between the number of carious surfaces and the time required to reach the end point was found possible.

Assay of Amylase Activity by Determination of Total Reducing Sugars Formed.—Since we found the qualitative determination of amylase activity by the spot-plate method unsatisfactory, we attempted to devise a quantitative procedure that would be fairly simple, yet accurate and capable of duplication. The procedure finally adopted was that of a serial dilution

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of saliva incubated with starch under standard conditions, and estimation of the total reducing sugar as glucose by the Folin-Wu method.[7] A procedure, involving the starch-iodine end point and using the same serial dilution, was also developed. All individuals studied were determined to be caries free or the number of carious surfaces was counted by visual examination and confirmed by x-ray examination.*

Reagents:

1. Starch solution: suspend 1.0 Gm. of soluble corn starch in about 80 c.c. of distilled water. Warm on a water bath with occasional stirring until a clear solution is formed. Transfer to a 100 c.c. volumetric flask, dilute to volume, and store in the icebox.

2. Iodine indicator solution; weigh approximately 1.2 Gm. iodine and dissolve it in 80 c.c. water containing 1.80 Gm. potassium iodide. Transfer to a 100 c.c. volumetric flask, and dilute to volume. The 0.1 N iodine solution should be stored in a dark bottle.

3. Salt solution; dissolve 0.9 Gm. NaCl in 100 c.c. water.

4. Buffer pH 6.6; to 50 c.c. of 0.2M acid potassium phosphate, KH_2PO_4, add 17.80 c.c. of 0.2 N NaOH and dilute to 200 c.c. with distilled water.

5. 0.1 N Hydrochloric acid.

Method:

1. Collect about 5.0 c.c. saliva, either with or without chewing paraffin. Saliva was always collected about two hours after eating.

2. Label a series of ten test tubes, 0 to 9, the zero tube serving as a control; place in each tube 1.0 c.c. of salt solution.

3. Add 1.0 c.c. saliva to Test Tube 1, mix, transfer 1.0 c.c. to Tube 2, mix, transfer 1.0 c.c. to Tube 3, mix. Continue to Tube 9, and discard the last c.c. of saliva mixture. There are now 9 tubes of serial diluted saliva.

4. To each test tube add 1.0 c.c. of the buffer pH 6.6.

5. Bring all the test tubes and the 1 per cent starch solution to 37° C.

6. Add 1.0 c.c. of the starch solution to each tube.

7. Mix and incubate for ten minutes at 37° C.

8. Stop enzyme activity by immersing the tubes in ice water and rapidly adding 2.0 c.c. of the 0.1 NHCl to all of them.

9. Transfer 1.0 c.c. of the contents of each tube to Folin-Wu sugar tubes and proceed as usual in determining the reducing capacity of each tube. Tube 0 serving as a control.

The serial dilution of the saliva permits the assay of amylase activity against concentration, with pH, temperature, and time kept constant. If we are actually measuring enzyme activity, the curve obtained by plotting the glucose found should be a straight line indicating that the activity is directly proportional to the concentration of the enzyme. Further, if this can be demonstrated, it then will be possible to select any dilution and to run a series of individuals and determine their amylase activity in terms of glucose equivalents. To test these postulates, a series of experiments were run upon noncarious individuals. The saliva was collected two hours after breakfast for at least six days and analyzed as described. A typical set of results is given in Table I. Tubes 1, 3, 5, 6, and 7 were run and the values for the glucose equivalents as mg. of glucose were determined. The averages for the individual tested and for four others are given in Table II, together with the number of determinations upon which the averages are based.

For comparison we have included the average of a similar series of determinations upon the saliva of twenty-seven individuals with caries in varying

*The examinations were made by senior dental students under the supervision of Joseph J. Kelly, D.D.S., Professor of Operative Dentistry.

TABLE I

MILLIGRAMS GLUCOSE FOUND IN SERIALLY DILUTED SALIVA INCUBATED FOR TEN MINUTES WITH STARCH

DAY	TUBE NUMBER				
	1	3	5	6	7
1	4.10	3.66	3.30	3.20	2.20
2	2.90	3.10	2.58	2.20	1.80
3	3.20	3.60	3.00	2.80	1.90
4	2.50	2.50	2.50	2.00	1.40
5	2.80	2.40	2.50	2.20	1.60
6	2.70	2.70	2.20	2.10	1.80
7	3.30	3.30	2.77	2.56	1.89
Average	3.04	3.04	2.60	2.44	1.80

TABLE II

AVERAGE MILLIGRAMS GLUCOSE FOUND IN SERIALLY DILUTED SALIVA INCUBATED FOR TEN MINUTES WITH STARCH

NUMBER OF DETERMINATIONS	TUBE NUMBER				
	1	3	5	6	7
7	3.04	3.04	2.60	2.44	1.80
5	3.00	2.60	2.30	1.65	1.12
5	2.95	2.67	2.01	1.63	1.02
5	3.83	3.10	2.52	1.69	0.98
5	3.07	2.67	2.33	2.00	1.45
General Average	3.18	2.82	2.35	1.88	1.27
Average of 27 Carious Individuals	3.44	2.91	2.39	1.84	1.38

degrees. In Fig. 1 both sets of averages taken from Table II are plotted. The points are close together, and a straight line common to both can be plotted. It is apparent that the method is measuring enzyme activity, and that the activity is directly proportional to the concentration. With these points established, we ran all the individuals at serial dilution corresponding to Tube 7.

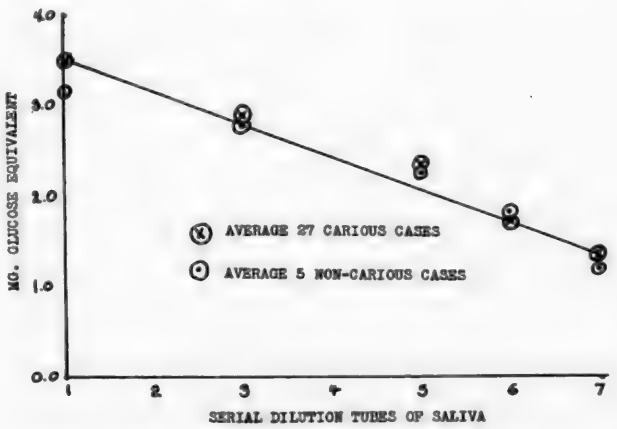

Fig. 1.—Effect of concentration of salivary amylase on starch hydrolysis.

In Table III is presented the data on both carious and noncarious individuals for the activity of the amylase as measured by the reduction in dilution Tube 7 (Columns 3 and 6). The glucose equivalent for the twenty noncarious individuals was 1.45 mg. with a standard deviation of ± 0.57. For the thirty-six carious individuals the glucose equivalent was 1.38 mg. with a standard deviation of ± 0.69. Calculation of the t. value gave 1.1, indicating that there is no significant difference between the amylase activity in the salivas of carious and noncarious individuals.[8]

TABLE III

AMYLASE ACTIVITY OF THE SALIVA OF CARIOUS AND NONCARIOUS INDIVIDUALS BASED UPON THE MILLIGRAMS GLUCOSE EQUIVALENTS IN SERIAL DILUTION TUBE 7 AND BY THE IODINE METHOD IN SERIAL DILUTION TUBES

1	2	3	4	5	6	7
NONCARIOUS			CARIOUS			
AGE	MG. GLUCOSE	I_2 METHOD TUBE NO.	AGE	NO. OF CAVITIES	MG. GLUCOSE	I_2 METHOD TUBE NO.
x*	0.98	6	7	16	1.43	7
x	1.78	6	15	9	1.11	7
x	0.94	7	16	40	1.97	6
x	1.24	7	15	20	0.60	8
x	0.98	6	x	20	1.65	8
x	0.78	6	16	40	2.20	8
18	1.45	7	10	2	1.98	9
18	1.51	7	17	20	0.60	6
19	2.51	9	19	20	1.02	7
19	2.40	8	x	20	1.95	8
x	1.94	8	11	6	1.86	9
x	1.65	8	15	20	1.92	8
19	1.07	7	16	20	2.28	9
x	1.41	7	16	15	2.79	9
x	2.04	8	16	14	3.24	9
x	1.08	3	x	5	1.32	7
x	0.40	4	9	11	0.37	6
x	0.92	7	13	14	2.46	8
x	1.93	9	16	14	0.81	7
x	2.07	9	16	40	1.02	6
			16	40	0.52	6
			x	10	0.60	7
			x	10	1.05	7
			18	20	1.38	5
			x	3	1.50	7
			x	20	0.24	7
			19	9	1.25	7
			12	10	1.11	6
			18	12	0.60	7
			18	14	0.75	7
			x	20	0.93	7
			x	20	1.62	7
			x	17	1.38	7
			x	20	1.65	7
			x	12	1.44	8
			x	20	1.20	8
Average	1.45	6.95			1.38	7.22
S_d	0.57				0.69	

*x = over 21 years.

Iodine Color Test in the Serial Dilution Tubes.—Since only 1.00 c.c. of each serial dilution was needed for the Folin-Wu method, we used the remaining solution to test further the validity of the starch-iodine method. To each serial tube remaining after the 1.00 c.c. had been removed, there was added one drop

of the 0.1 N iodine solution and the colors noted. The tube in which an orange or yellow color first appeared was taken as representing complete hydrolysis of starch. The results by tube, for twenty noncarious individuals are given in Table III, Column 3, together with the results of thirty-six carious individuals, Column 7, with their ages and caries count. It is evident that there is no difference between the activity of the saliva of normal and of carious persons as measured visually by the iodine color method.

DISCUSSION

The activity of salivary amylase has been determined by the quantitative estimation of the glucose equivalent of the reducing sugar formed. It has been shown that salivary amylase activity is directly proportional to concentration by making estimations upon serial dilutions of saliva incubated with starch for ten minutes at pH 6.6 and at a temperature of 37° C. Repeated determinations of salivary amylase activity upon the same individual revealed no significant differences from day to day. When the amylase activity of a series of twenty noncarious individuals was determined and compared with that of a series of thirty-six individuals with caries in varying degrees, no significant difference was found.

While this study was in progress a report by Barany[9] appeared in which essentially the same conclusion was reached. Barany also measured amylase activity by a determination of reducing sugars formed; however, he used time rather than concentration as the variable. The activity of enzymes varies with pH, temperature, time, and concentration. Barany and we maintained the same pH and temperature, and differed in the third variable measured. It is significant, therefore, that using either time or concentration as the variable factor, no significant differences were found between the salivary amylase activity of carious and noncarious individuals.

When the starch color test is employed upon aliquots of the same serial dilution of saliva incubated with starch, a qualitative estimation of digestion is possible. If it is assumed that the disappearance of the blue color has significance and the tube noted which exhibits only a yellow or orange color, a comparison can be made between the carious and noncarious individuals. When this was done, no significant difference could be found.

CONCLUSIONS

Salivary amylase activity can be measured in serially diluted saliva by the quantitative estimation of total reducing sugar by the Folin-Wu method. Amylase activity is directly proportional to concentration when pH, temperature, and time are kept constant.

Salivary amylase activity in the same individual does not vary significantly from day to day.

There was no significant difference between the salivary amylase activity of a series of twenty noncarious individuals and a series of thirty-six individuals with caries of varying degrees.

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STUDIES OF THE BACTERIAL PLAQUE AND DENTAL CARIES

I. Method for the In Vivo Production of Bacterial Plaques Suitable for Microsectioning

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INTRODUCTION

IN A long-term plan for investigation of dental caries, it was deemed essential that the bacterial plaque should be systematically studied.[1] Beust,[2] in 1912, called attention to the need for "sections through the bacterial plaques found in nature . . . coupled with careful microscopic analysis." The need for these sections is still present because the thread forms in the plaque usually fragment when prepared for study as smears. Occasionally, decalcified sections of tooth structure may include remnants of plaque structure, and material suggestive of the plaque may be found on some ground sections. However, even in the most fortuitous of these preparations, the plaque is markedly altered, so that their value for study is dubious.

It has been shown in this laboratory[5] and elsewhere[3, 4] that bacterial plaques which are qualitatively similar to those in the mouth can be produced on various surfaces. Celloidin may serve as a surface for plaque formation.

EXPERIMENTAL

Preparation of Experimental Denture.—A removable appliance was constructed for a region in which a single tooth was missing. The interproximal surfaces of the adjacent teeth were not covered by the denture frame. A paper matrix was adapted to the pontic area on the appliance and 15 per cent celloidin was poured in, care being exercised to keep air bubbles at a minimum. The celloidin block was hardened in air under an inverted staining jar. After final hardening, the block was carved to approximate tooth size and shape. Treatment with ether alcohol and neutralization with absolute alcohol afforded very smooth surfaces for the celloidin pontic. The denture with the pontic was then left in 40 per cent alcohol for approximately twenty-four hours before insertion in the mouth.

Exposure of Pontic to Oral Environment.—Subjects wear the dentures for specified periods of time, at the end of which interval the entire denture is placed in Bouin's Picro-Formal fixative, shaken to remove extraneous debris, and transferred to fresh fixative.

Preparation of Pontic for Sectioning.—The celloidin pontic is partially dehydrated by the denture being carried through a series of alcohols from 40 per cent to 80 per cent over a period of forty-eight hours. Final dehydration is accomplished by two twenty-four hour changes of propyl alcohol over a hydrous copper sulfate in a desiccator. The denture is kept in the final propyl alcohol desiccation for approximately forty-eight hours.

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When dehydration has been completed, the celloidin pontic is removed from the denture frame by cutting the pontic mesio-distally through the center of the occlusal surface and lifting each portion away by means of a straight pin inserted at a right angle to the buccal or lingual surface. The separated pieces of celloidin are then placed, cut surface down, in a 100 ml. jar and embedded in 10 per cent celloidin. The pins serve to stablilize the material and are not removed until the embedding celloidin had been allowed to harden again under an inverted staining jar. When the newly added celloidin has assumed a rubbery consistency, the embedding blocks are removed from their jars and immersed in chloroform until sectioned.

Sectioning and Staining.—The blocks containing the pontic and its plaques are sectioned on a sliding microtome and stained by a modified Gram-Weigert method.[6]

Fig. 1.

DISCUSSION OF RESULTS

Fig. 1 illustrates a section of plaque produced on a denture in thirty hours and cut at approximately twenty microns. Although the density defies any attempt at differentiation, the typical surface structure is readily noted. Fig. 2 is a section of the same plaque cut at ten microns. The heavy dark area is the attachment to the celloidin (*A*). There are a multitude of filamentous

forms, roughly parallel to each other and approximately at right angles to the attachment visible in the body of the plaque (*B*). Clumps of other bacterial forms appear supported by the filaments among which they are seen. Fig. 3 is a photomicrograph of a nineteen-hour plaque cut at eight microns. The celloidin is at *A* and the line of plaque attachment extends obliquely through *B*. This section demonstrates that the thread forms, such as seen in the body of the plaque in Fig. 2, also occur at the junction of plaque with solid surface. The threadlike projections into the celloidin are artifacts.

Fig. 2.

These sections afford material for study of the plaque in situ. The chief advantage of the method is in supplying relatively undisturbed plaque structure in its natural habitat. We are attempting to improve the technic, here described in the hope that more critical analyses will be possible. These efforts are being correlated with cultural studies.

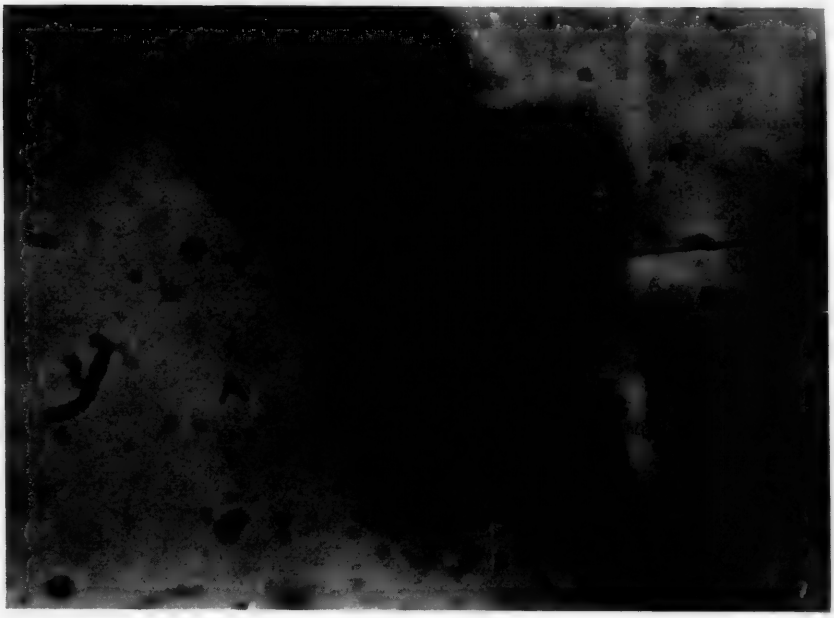

Fig. 3.

SUMMARY AND CONCLUSIONS

The bacterial plaque, which is believed essential for the initiation of caries, has been produced on a celloidin "tooth" in the mouth and has been sectioned without disturbing its relationships. The thread forms are apparent and related cultural studies are in progress.

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VARIATIONS IN SALIVARY PROTEIN CONCENTRATION

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THE purpose of this study was to investigate further the variation in salivary protein concentration in different individuals. This study was necessary because of variability in both pattern and mobility of the several components obtained by means of the Tiselius electrophoretic apparatus. It was thought worth while to determine whether constant amounts of salivary protein were secreted by different individuals when the many variables entailed in collection technic and treatment before and during analysis were made constant.

Krasnow and Oblatt[1] determined the ratio between tyrosine and protein to be 1 to 21. They modified the total serum-protein method of Greenberg[2] for salivary protein analysis. This method was based upon the measurement of the amount of tyrosine present in the sample, the protein being assumed to be a proportional function of the amount of tyrosine in the sample. Analyses were made on saliva of caries-immune and caries-susceptible individuals. Frequency curves of this study led the authors to believe that (1) higher salivary protein values occurred more often in caries-immune individuals; (2) during the day, salivary protein of caries-susceptible individuals was subject to less change; (3) on centrifugation, more protein was sedimented from saliva of caries-susceptible subjects, suggesting that possibly salivary protein of caries-immune persons is more stable. Krasnow and Oblatt found most marked, direct correlation between pH, phosphate, and titratable alkalinity in morning unstimulated samples.

The method of Greenberg was also utilized for estimation of salivary protein in the studies of Karshan[3] and Tenenbaum and Karshan[4, 5] in their correlation investigations of salivary factors in relation to caries and calculus deposits.

Karshan[3] presented values of salivary protein found in four groups: caries-free, caries-arrested, caries-active, and a miscellaneous group. His findings are summarized in Table I.[3]

TABLE I

CLASSIFICATION	NUMBER OF CASES	$\overline{X}$ MG. %	σ MG. %	σ_m MG. %	CLASS $\overline{X}$ MG. %
Calculus-free	22	266	44	9.6	
Caries-arrested	15	285	51	13.5	286
Caries-active	39	283	52	8.5	
Miscellaneous	26	310	85	17.2	

$\overline{X}$ = Mean σ = Standard Deviation σ_m = Standard Deviation of the Mean.

Karshan concluded that there were no significant differences among the four groups.

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From studies of Tenenbaum and Karshan[4] on the correlation of factors in stimulated saliva with the occurrence of calculus, the values shown in Table II were found.

TABLE II

CLASSIFICATION	NUMBER OF CASES	$\bar{X}$ MG. %	σ MG. %	σ_m MG. %	CLASS $\bar{X}$ MG. %
Calculus-free	20	314	75.5	17.3	302
With calculus	29	294	69.9	13.2	

While the mean value of salivary protein was higher in the calculus-free group than in the group with calculus, the wide range within the groups caused the values to have no statistical significance.

Analyses of unstimulated saliva by Tenenbaum and Karshan[5] revealed the facts found in Table III.

TABLE III

CLASSIFICATION	NUMBER OF CASES	$\bar{X}$ MG. %	σ MG. %	σ_m MG. %
Calculus-free	18	298	77	18.7
With calculus	28	348	117.8	22.7

The authors[5] concluded that there was no significant difference between the groups in stimulated and unstimulated saliva.

Brown and Kletz[6] collected saliva from 88 first-year medical students who chewed paraffin for two consecutive fifteen-minute periods, and then measured the amount of saliva collected during that time. These investigators found that the mean volume obtained during the first period was 36 c.c., during the second period, 31 c.c., and during the two periods, 67 cubic centimeters. The range of values was 63 c.c. to 138 cubic centimeters. General constancy of secretion values from day to day was noted. The students were divided into three groups on the basis of the volume of saliva collected. Eighteen men were chosen and segregated on different mornings, and stimulated saliva was collected for one hour. Total nitrogen was estimated by the micro method of Folin and Wu.

Analysis of their data reveals that total nitrogen of the "low (volume) producing" group was decidedly higher than that of the two other groups. No attempt was made to determine whether this was due to protein or nonprotein nitrogen. The mean value of the "low group" was 57.5 mg. per cent, the "medium group" was 37.4 mg. per cent, and the "high group" was 43.3 mg. per cent; the standard deviations for these series calculated by the present author are 13.6, 10.7, and 11.2 mg. per cent, respectively.

Becks and Wainwright[7] called attention to the need of uniformity in the preliminary treatment of saliva after collection and before proceeding with any chemical analysis. The authors have shown, for example, that centrifugation, deproteinization, or both, produce errors in calcium determination on whole saliva. Centrifugation alone decreased the average amount of calcium 6 per cent when analysis was carried out on the same sample by the same method by

the same investigator. The etiology of this decrease was inferred to be the removal of some of the salivary protein by centrifugation.

The effect of storage temperature upon the precipitable protein content of whole saliva was studied by Deakins.[8] Salivary protein may be decomposed by either proteolytic enzymes, the existence of which in saliva has been shown by Warfield[9] and Voss,[10] or by bacteria. While the action of the latter may be inhibited by toluene or chloroform, they exert no influence upon the enzymatic action that takes place. Deakins concluded from this study that the amounts of protein nitrogen and nonprotein nitrogen were appreciably affected by storage at room temperature. The protein content of whole saliva decreased 42 per cent on standing for 24 hours at 30° Centigrade. The protein content of centrifuged saliva decreased 34 per cent under the same conditions. He found that no significant change took place at 0° Centigrade. Only a small change was noted within the first four hours after standing at 30° Centigrade.

In a study by Deakins, Cheyne, Bibby, and Van Kesteren,[11] salivary protein was determined by the Pregl method in mg./c.c./min. Samples were obtained from 20 healthy subjects who had no serious dental defects; 13 were laboratory workers and 7 were high-school students. Stimulated whole saliva was obtained by the subjects chewing paraffin, and collection was made in iced 50 c.c. centrifuge tubes. Samples were analyzed immediately. The age range was from 12 to 33 years. The range of salivary protein nitrogen was 2.80 to 29.41 mg./c.c./min. The protein nitrogen in mg./c.c. was 22.9 to 88.2. When divided into "fast" and "slow" groups of eight each, there was statistical significance found between the amount of salivary protein nitrogen in mg./c.c. and the rate of flow.

METHOD

In this study a number of variables in the collection technic and treatment of saliva before and during analysis were made constant, and every attempt was made to minimize protein loss through enzyme action.

The size of the paraffin block was uniform in all cases ($1 \times 1 \times \frac{1}{2}$ cm.); the time of salivary stimulation previous to collection was two minutes; the amount of saliva collected was five cubic centimeters; the time of centrifugation was five minutes; the collection analysis interval was one-half to one hour. Salivary samples were collected from 35 volunteers, 24 of them were medical students, and 11 were laboratory workers and office help about the dental school. All of the volunteers had teeth in good repair. The individual mouths were not charted for caries and calculus occurrence nor was the systemic condition of the volunteers taken into consideration. For uniformity in protein intake, volunteers were selected from a group of individuals that did not have breakfast on the morning of collection. Centrifuged saliva was used to cancel the individual differences in salivary debris. The effect of smoking and water intake upon salivary protein concentration was not determined in this study. Because individuals secrete saliva at different rates, the volume limit of 5 c.c. rather than a time limit was utilized. Women volunteers were requested to remove lipstick before collection. The age range was from 19 to 37 years.

The method of Greenberg[2] for total serum protein, as adapted for salivary protein analysis by Krasnow, was employed. One c.c. aliquots were placed in 25 c.c. volumetric flasks after centrifugation. One c.c. of 5 N potassium hydroxide and 10 c.c. of distilled water were added and agitated by rotating the flask. One and one-half cubic centimeters of phenol reagent were added slowly with agitation to prevent cloudiness. Distilled water was then added to the 25 c.c. mark. The flasks were then shaken and allowed to stand for 10 to 15 minutes, to insure full development of the blue color. The samples were then read on a B.&L. colorimeter using 1 c.c. of a 0.2 mg./c.c. tyrosine solution treated in a similar manner as a standard.

$$\text{Salivary protein} = \frac{S}{U} \times 0.2 \times 21 \times 100 \text{ mg. \%}$$

S = Colorimeter reading of standard
U = Colorimeter reading of salivary sample

RESULTS

TABLE IV

NUMBER OF SAMPLE	SALIVARY PROTEIN MG. %	NUMBER OF SAMPLE	SALIVARY PROTEIN MG. %
1.	318	19.	175
2.	344	20.	200
3.	254	21.	210
4.	247	22.	373
5.	262	23.	442
6.	249	24.	365
7.	240	25.	420
8.	300	26.	254
9.	254	27.	254
10.	253	28.	382
11.	316	29.	191
12.	210	30.	262
13.	323	31.	210
14.	323	32.	262
15.	262	33.	247
16.	262	34.	382
17.	210	35.	234
18.	323		

$$\text{Mean equals } \bar{x} = \Sigma \frac{\times}{N} = \frac{9813}{35} = 280.4 \text{ mg. \%}$$

Standard deviation $= \sigma = 65.93$ mg. %

The range of values was 267 mg. per cent

SALIVARY PROTEIN MG. PER CENT	NUMBER
140-180	1
181-220	6
221-260	10
261-300	6
301-340	5
341-380	3
381-420	3
421-460	1

$\bar{x} \pm 1\,\sigma = 214.5 - 346.3$ mg. %
23 values (65.7%) are within 1 σ.
It may be said that the values obtained follow roughly a normal curve distribution.

Fig. 1 shows the histogram of frequency distribution. The mean of this series was 280.4 mg. per cent of salivary protein. The standard deviation was 65.93 mg. per cent.

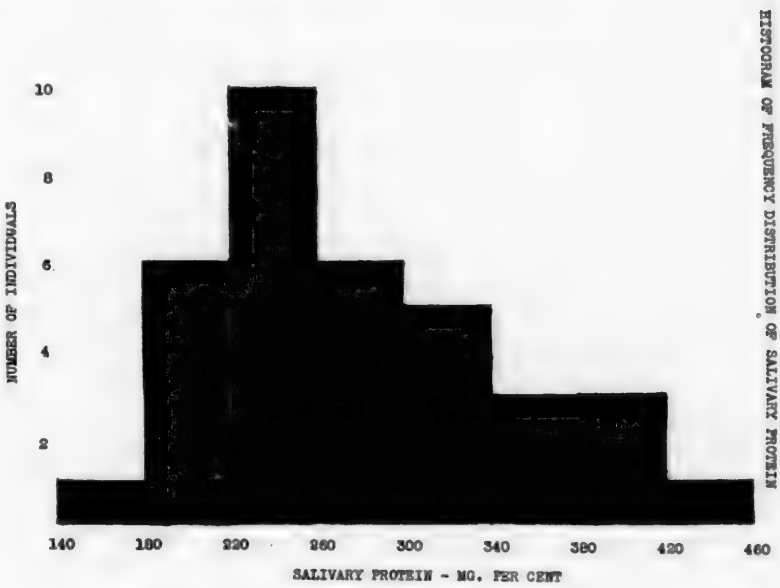

Fig. 1.

CONCLUSIONS

It may be concluded from this study that the amount of salivary protein secreted by different individuals is highly variable. The wide range of values is manifest even when there is uniformity of collection technic and treatment before and during analysis, with utmost care being taken to minimize protein loss through enzymatic action.

SUMMARY

Fasting whole saliva was collected from 35 individuals to determine whether constant amounts of salivary protein was excreted when there was uniformity in collection technic and treatment before and during analysis. Utmost care was given to prevent loss of protein through enzyme action.

The method of Greenberg, as adapted for salivary protein by Krasnow and Oblatt, was utilized on 1 c.c. samples. A mean value of 280.4 mg. per cent was obtained. The standard deviation of the series was 65.93 mg. per cent.

It may be concluded that control of the factors introduced in this study had no effect upon the variability of salivary protein concentration.

The author is greatly indebted to Dr. Maxwell Karshan for his advice and encouragement in this work.

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THE ORGANIC ELEMENTS OF THE ENAMEL

I. A Study of the Principal Factors Involved in the Histological Preservation of the Organic Elements of Enamel and Other Highly Calcified Structures

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HISTOLOGICAL information about the dental enamel has primarily been gained through the study of ground sections, a wider application of which has recently been suggested (Sognnaes, 1947). Ground sections are, however, of limited value for the histological study of the organic elements of enamel. Neither do other routine histological procedures suffice for a separate sectioning of the organic framework, which, as is well known, is extremely difficult to isolate from all highly calcified structures. Indeed, when exposed to decalcification, in the course of a standard hard tissue preparation, the fully formed enamel, contrary to dentin and bone, disappears as if it were nothing but a "solid, dense, adamantive, inorganic mass." (Hopewell-Smith, 1931). This is not, as is often assumed, convincing evidence against the presence of organic elements in mature enamel. Occasional results of careful histological procedures suggest indeed that the general failure to demonstrate organic structures in fully formed enamel may well have been due to shortcomings of conventional technics rather than to biological peculiarities unique to the enamel. The following analysis has been made in the belief that further progress is possible through a better understanding of the nature of the technical difficulties and the principles involved in the successful isolation of organic elements from highly calcified structures in general and the enamel in particular.

PRINCIPAL FACTORS INVOLVED

An attempt has been made to analyze the principal factors which, in the past, appear to have contributed to a more or less satisfactory recovery of organic elements in decalcified sections of enamel and certain other highly calcified structures. In Table I the different contributions have been listed chronologically with emphasis on what appears to be the first introduction of a new technical approach. The essential nature of each step of the technics will be discussed under three major headings: (1) fixation; (2) immobilization, and (3) decalcification, which from Table I appear to be subject to the most crucial modifications.

1. *Fixation.*—For the successful recovery of organic elements from the enamel, Abbott (1879) recommended the use of fresh tissue, that is, unfixed enamel, while Malleson (1924) and others have emphasized the need of a prolonged fixation. These very opposite approaches immediately raise the question whether their results were due to their modification of the fixation procedure *per se*, or unintentionally caused by other features of their technic. The latter is suggested from the following theoretical considerations.

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TABLE I

PROCEDURES CONTRIBUTING TO THE HISTOLOGICAL PRESERVATION OF ORGANIC ELEMENTS IN MATURE ENAMEL

AUTHOR	MATERIAL	METHOD	REMARKS ON RESULTS
1. Waldeyer, C. 1872	Enamel pieces	Decalcification in *dilute acid* (HCl)	Isolated prisms; cross striations.
2. Wedl, C. 1872	Enamel pieces	Same but independently of above	Suggests that the amount of organic material determines color of enamel.
3. Koch, G. 1878	Ground sections of corals mounted on slides with balsam	(a) *Careful decalcification* and staining of (b) *immobilized* specimen on slide	Limited to single, thick section.
4. Abbott, F. 1879-80	Ground sections of freshly extracted teeth	*No fixation.* Dilute acid (0.5% chromic)	"Delicate reticulum of organic matter."
5. Bödecker, C. F. W. 1878-79	Ground sections of freshly extracted teeth	Credits method to No. 4	Shows drawings of prism sheaths, cross striations, and "interstices."
6. Weld, G. W. 1886	Ground sections of freshly extracted teeth	(a) Minimum fixation (b) Decalcification in weak *acetic acid* (6%)	Believes this acid has a "selective action on interprismatic substance."
7. Abbott, F. 1887	Ground sections of freshly extracted teeth	As No. 4 and No. 6. First description of various stages of decalcification	Distinguishes reticular basis substance, enamel fibers, "elastic ledges," (prism periphery), transverse threads of prisms, and wavy course of prisms.
8. Vosmäer, J. 1887	Calcareous sponges	(a) Slow dropwise decalcification in acetic acid (b) Paraffin embedding	Thin sections of organic remains.
9. Kultschitzky 1887	General for delicate tissue	Double embedding in celloidin and paraffin	Thinner sections than with celloidin alone.
10. Thoma, R. 1891	General	Decalcification in *acid* alcohol. *Neutralization* in carbonated alcohol	Effective decalcification; reduced swelling; good staining.
11. Rousseau, E. 1897	Ground slabs of calcareous sponges	(a) *Pre-embedding in celloidin* (b) Decalcification as No. 10	Fairly thick sections
12. Miller, W. D. 1902	Ground sections of enamel	Decalcification *under* the microscope with dilute acid (2% HCl). Similar to No. 3	First illustration of enamel "lamellae." Interprets organic film on decalcified ground surface of the enamel as the true basis substance of enamel.
13. Andresen, V. 1902	Whole teeth	*Simultaneous fixation and decalcification* (formalin and HNO_3) of *suspended specimen*	First photographic reproduction of organic framework in decalcified sections of enamel.
14. Schaffer, J. 1902	Delicate calcified structures	Pre-embedding in celloidin as No. 11. Fixation-decalcification (5% HNO_3 in 10% formalin). Neutralizing (5% sodium sulphate). Fundamental study of decalcification process	Minimum swelling and good staining obtained.

TABLE I—CONT'D

AUTHOR	MATERIAL	METHOD	REMARKS ON RESULTS
15. Bödecker, C. F 1905, 1906, 1908, 1909	Small pieces of enamel	(a) Simultaneous decalcification and embedding in celloidin-concentrated HNO_3 mixture (b) Re-embedding in paraffin as No. 9	"Very involved and time taking" (1909). Rediscovers Linderer's (1843) "Buschel's" (Tufts) and Miller's (1902) "Fasern" (lamellae).
16. Breckner, A. 1908	Delicate and highly calcified structures	(a) Pre-embedding as No. 11 (b) Uninterrupted submersion in fluid and immobilization in double glass cylinders (c) Re-embedding in paraffin as in No. 9	Thin serial sections.
17. Fleischmann, L. 1908	Ground sections of enamel	(a) Embedding as No. 11 (b) Decalcification in 5% HCl	No illustrations.
18. Baumgartner, E. 1911	Whole enamel	As No. 15, using slower-hardening acid *Methyl-alcohol-celloidin* mixture	Serial sections, but incomplete preservation.
19. Rygge, J. 1916	Ground section of whole teeth with soft and hard structures	Adaptation of No. 3 and No. 12	First demonstration of the progression of calcification of the enamel from cusps to neck of teeth.
20. Jenkins, C. E. 1921	General	*Simultaneous decalcification* and *dehydration* (acid alcohol, similar to No. 10)	Prevents accidental stains; aids selective staining.
21. Malleson, H. C. 1924 1925	Pieces of enamel	(a) Prolonged fixation (b) Celloidin embedding (No. 11) (c) Simultaneous decalcification and fixation (No. 13) (d) Re-embedding in paraffin	Serial sections of well-preserved and more completely decalcified organic elements.
22. Kato, S. 1928	Ground sections of enamel on slide	(a) Coated with celloidin (b) Decalcification in *citric acid*	A few apparently incompletely fixed and decalcified sections.
23. Cape, A. T., and Kitchin P. C. 1930	Ground sections of enamel on slide	Decalcification under the microscope in acid celloidin (combining Nos. 3, 12, 15, and 19)	Single thick sections.
24. Arnim, S. S. 1935	Rat jaws	(a) Acid fixation (b) Prolonged dehydration (c) Pre-embedding as in Nos. 11 and 16, but more prolonged (d) Decalcification as No. 6 (7%) (e) Re-embedding in paraffin (as No. 9)	Very slow. Preservation of surface and carious areas.
25. Frisbie, H. E., Nuckolls, J., and Saunders, C. M. 1944	Small pieces of enamel	(a) Prolonged fixation (as No. 21) (b) Simultaneous decalcification and fixation (as Nos. 13, 21) (c) Embedding in nitrocellulose	Very slow. Prism sheaths obscured.
26. Forshuvud, S. 1944	Whole teeth	(a) Simultaneous fixation and decalcification (as in Nos. 13, 14, and 21) under four atmospheres pressure (b) Immobilization (as No. 16) (c) Celloidin embedding (d) Redecalcification (e) Re-embedding	Isolation of well-defined interprismatic heaths. Interprets certain staining reactions as reticulin fibers, "para capillaris," and nerve fibers in the enamel.

In view of the low permeability of morbid enamel, it would seem unlikely that the ordinary fixatives which have been used in the past can be of much benefit to the highly calcified organic framework. Enamel may be preserved in dry condition without such fixatives for thousands of years. During excavations of prehistoric graves, the enamel caps of teeth are sometimes the only human remains scattered among the tools of early man (Fig. 1). Indeed, very similar "remains" can be obtained in vitro in one-half hour by the boiling of teeth in 50 per cent sulphuric acid. This appears to dissolve all tissues with more readily accessible organic matter such as bone, cementum, and dentin, but it leaves a shell of enamel grossly intact (Fig. 2). Such observations seem to suggest that most of the organic elements of the enamel are so encapsulated in and infiltrated by highly calcified matter that they are virtually inaccessible to proteolysis and chemicals before the mineral components have been acted upon. At least, this is to be expected in the morbid state with which histology is concerned. The fact that ground sections of the enamel are extremely difficult to stain does not, as is also often assumed, mean that enamel contains little or no organic matter, but it is further evidence of the nearly complete mineralization of the organic elements. Were they not so highly calcified, the bulk of the organic elements, as observed in decalcified sections prepared under special precautions by the author (Figs. 3, 4, and 5), would show up as a considerable weight loss upon ashing. In reality the organic component rarely amounts to more than 5 per cent by weight.

Enamel affected by *in vivo* decalcification or caries, or by *in vitro* changes caused by acid preservatives, might, on the other hand, be expected to gain a considerable degree of fixation, especially if these naturally or artificially demineralized organic elements are exposed to fixatives over a long period of time. This reasoning is indeed supported by studies of enamel affected by caries, because relatively little precaution is usually necessary for the successful preservation of the rigid organic elements frequently contained in and surrounding such lesions. Although most of the enamel may be destroyed in the routine decalcification of teeth, these carious areas are the last ones to be lost unless torn off by careless handling. Moreover, if the enamel is hypocalcified due to developmental disturbances, the organic elements may even be preserved with routine technics. It is to be expected that such abnormal or changed enamel which affords access to its organic constituents will be benefited by fixatives in the same manner as any other organic structures. But for normal, mature, intact enamel, with which the present problem is concerned, it is felt that the fixation should be obtained by more rational means than the mere expenditure of time. Fixation for one-half year as suggested by Malleson (1924), and recently re-emphasized by Frisbie, Nuckolls, and Saunders (1944), does not appear particularly rational from the above considerations.

If this reasoning is correct, then the simplest approach to fixation of enamel would be to employ a "fix-as-you-go technic," that is, a combination of fixative and acid rather than a neutral fixative. By such a combination, fixation can be obtained successively because the organic matter is freed from minerals. It is believed that this principle rather than the use of "freshly extracted teeth"

explains Abbott's and Bödecker's success with chromic acid which, in itself, is a good protein fixative, in addition to being a satisfactory decalcifier, for which it was intended. It is also noteworthy that Malleson, as well as Frisbie, Nuckolls, and Saunders, and several others before their time, used a combined decalcification-fixation procedure in addition to the prolonged prefixation.

From these considerations it is suggested that fixation *per se* if actually achieved may well be of very great importance to the successful preservation of the organic framework of mature normal enamel, but so far no one has convincingly shown this, and no method has as yet been originated which has satisfactorily met the practical and theoretical difficulties inherent in the proper prefixation of this highly mineralized structure. Preliminary results in this laboratory suggest that prefixation of the enamel protein may be achieved by heat; this in principle is one method not requiring direct penetration or access to the encapsulated mineralized organic framework of enamel.

2. *Protection of the Specimen* (*Immobilization*).—One feature, intentional or not, of nearly all of the more successful methods referred to in Table I, is an immobilization of the highly calcified specimens, particularly during the process of decalcification. Koch (1884), studying the highly calcified corals, obtained careful decalcification by first adhering one side of ground sections to a slide by means of balsam. Rousseau,[20] studying calcareous sponges, went a step further by enclosing the whole specimen in celloidin before removing the salts. Both of these procedures tended to immobilize the delicate organic framework as it became free of minerals. Most of the work on enamel has been done by Koch's, Rousseau's, or other similar principles. Andresen (1902), who was among the first to successfully preserve the enamel matrix, suspended his specimens in the center of his decalcification vessels. Suspension of the specimen in a double glass tube (Breckner 1908) has recently been utilized by Forshuvud (1944) to obtain satisfactory immobilization during decalcification. The pre-embedding of the enamel in a shell of celloidin, unsuccessfully attempted by Sudduth (1878), has by many others been found to afford a quite satisfactory protection during the whole process of decalcification, washing, and partial dehydration. Best known is probably Bödecker's method (1905) of immobilizing the organic elements by combining the decalcification and embedding agents, what may be referred to as an "embed-as-you-go technic." It goes without saying that any immobilization of the enamel by means of a medium (celloidin) which is insoluble in water must seriously affect the effectiveness of the acid, especially when only a few drops of the acid (HNO_3) were added in the concentrated form. It is, therefore, not surprising that the latter method required two months for the decalcification of a 1 mm.-thick chip of enamel. Aside from the historical interest, this method and similar ones (Baumgartner, 1911) do not seem to have a wide enough application to be a satisfactory goal for the future. Yet the principle of the method is both ingenious and attractive. If a water-soluble embedding medium (Lubkin and Carsten, 1944), the hardening of which is irreversible, can be applied to enamel, one might eventually succeed in developing a more effective "embed-as-you-go technic." In principle the need for immobilization of the delicate organic framework of the enamel is obvious. Without careful handling, no method could possibly succeed.

3. *Decalcification.*—A study of Table I shows that nearly all successful histological preparations of enamel have been based upon a modification of the process of decalcification. Several authors admit the accidental or empirical choice of their decalcification procedure, but a common principle seems to underlie the various approaches. If clearly understood, it should be possible to make a rational choice of future procedures.

The effect of dilute acid (HCl) on pieces of enamel as described by Wedl (1872) and Waldeyer (1872) was at that time not appreciated as a possible aid toward successful preservation of the organic elements of the enamel. Koch (1878), in his studies of the hard and highly calcified skeletons of marine organisms, must have employed careful decalcification of the exposed surface of ground sections to remove the carbonate of lime (deposited by the sea water) without breaking apart the delicate organic framework. But, relegated to a footnote, he did not state the type and concentration of the acid used.

The method of Abbott (1879-80-87) may be interpreted as a combination of the above principles. By a prolonged decalcification of ground sections in dilute acid (½ per cent chromic), he obtained the first known recovery of what he called "a delicate reticulum or frill of organic matter" in the enamel. The potentialities of his method were confirmed by Bödecker (1878-79) who was among the first to fully appreciate the significance of the observation. In his own more comprehensive report, Bödecker expressed his indebtedness to Abbott by whose method he had become able to recognize the so-called "enamel fibers." From Bödecker's detailed drawings, it appears that this term refers to the prism sheaths and not to the prisms themselves for which the term previously had been used (Wedl, Waldeyer). Abbott and Bödecker published sketches of several minute organic elements such as "nuclei" and "cross bars" in the prisms, and "branches" and "interstices" between the sheaths. Their published evidence was, however, questioned and the literature still lacks convincing photographic reproduction of some of the details they claimed to have seen. It is not surprising, therefore, that contemporaries failed to accept the conclusions based upon these early drawings which grossly were not unlike those of previously described ground sections. Sudduth (1886) who first, but without success, had tried decalcification of celloidin-embedded enamel, claimed that the dilute chromic acid only served to break apart the enamel prisms by first acting on the interprismatic substances. In his hands he claimed that "no trace of a matrix can be seen after decalcification."

Weld (1886) is given credit for having introduced a decalcification agent (acetic acid, 6 per cent) "by which anybody can demonstrate organic remains in enamel" (Abbott, 1887). After exposure to this acid, the enamel becomes soft and chalky, but retains its continuity. Weld attributed this phenomenon to a specific action of acetic acid upon the interprismatic substance which he believed was composed of carbonate of lime. He believed that the prisms were left relatively intact because they were composed of phosphate of lime. Such distinct difference in chemical composition of enamel and selective action of acetic acid was justly questioned by Heintzmann (1887) and by Abbott (1887). Nevertheless, it can easily be confirmed that acetic acid has a milder and less

destructive action upon enamel than the ordinarily used decalcification agents. This property has been taken advantage of in a recent method (Arnim, 1935), in which Arnim, apparently without benefit of above references, selected acetic acid of a concentration within 1 per cent of the choice of Weld (1886).

In principle, the various procedures for demineralization of highly calcified structures may be interpreted as a slowing down of the decalcification process. This has either been achieved by the use of dilute acids (references Nos. 1, 2, 4, 5, 6, 7, 12, 19, 22, 24 of Table I), by slow dropwise addition of the acid (No. 8), by acid-alcohol (No. 10), by acid-celloidin combinations (Nos. 11, 14, 15, 16, 18, 21, 22, 23, 24), or by an increased atmospheric pressure (No. 26).

A study of the published illustrations of all the authors listed in Table I shows that relatively few have obtained complete preservation of sharply defined organic elements of fully formed human and animal enamel. While all the early workers, with the possible exception of Malleson (1924), in presumably some of their best photographic reproductions, seem to have recovered an almost homogenous, weakly stained outline of the enamel prisms, the illustrations of Forshuvud (1944) show a definite, clearly defined, and well-stained organic framework, consisting mainly of the prism sheaths. Forshuvud decalcified his specimens in a pressure apparatus under 60 pounds pressure, that is, four times the atmospheric pressure. His report is mainly devoted to details of a complicated apparatus and the immediate histological findings.

To our discussion it is presently important to analyze the principles underlying Forshuvud's results. In the first place, his method is based upon a simultaneous fixation and decalcification (HNO_3 and formalin), a combination which has contributed to the success of many workers in the past his elaborate immobilization of the specimen is, furthermore, in principle at least, the basis for nearly all previous successes; moreover, a combination of all of these basic principles was the basis for Andresen's (1902) success years ago. Finally, Forshuvud's choice of the pressure of four atmospheres is said to have been based on trial and error. As a result, one cannot be sure that the pressure *per se* has been the most essential factor, and if so, it is not known where the critical pressure lies.

Before drawing any further conclusions on the merits of this newest method, it is therefore necessary to digress for a test of the action of pressure *per se*.

The miniature pressure decalcification apparatus shown in Fig. 6 was designed in this laboratory in order to allow observation of the specimen under a binocular microscope during the decalcification process. By means of this approach, it can be demonstrated that increased atmospheric pressure suppresses the formation of gas bubbles resulting from the chemical reaction between the acid and the enamel. This gas is mainly carbon dioxide formed by the action of acid upon the carbonates of the enamel. At thirty times magnification, visible bubbles tend to form at pressure below 10 pounds, and they are suppressed by pressure above 10 pounds. This holds true for the strongest acids (HNO_3) in the maximum concentration (10 per cent) necessary or desirable for enamel decalcification. If the pressure is brought to zero, that is, to normal atmospheric

pressure, one may observe grossly the formation of gas bubbles which first attach themselves to the surface of the enamel and soon start to escape from the interior of the enamel. When the surface cuticle has been decalcified, the bubbles attached to its surface and those accumulating below the surface tend to lift the organic cuticle from its normal relationship to the rest of the enamel. In this process, the cuticle is torn from the normal attachment which has been found to exist between the cuticle and the prism sheaths (Fig. 7). The normal relationship between the sheaths themselves is distorted and broken up by the further progress of acid and the concurrent formation of CO_2 in the internal enamel. Finally, the remaining delicate organic elements are torn from their attachment to the dentin surface by what appears to be a "balloon-action" of the numerous gas bubbles accumulating inside the surface cuticle. When escaping to the surface of the decalcifying fluid, these CO_2 balloons bring with them the delicate and light-weight organic elements.

Aside from the detrimental action of CO_2 itself, it seems moreover as though the organic membranes covering the external (Fig. 7) as well as the internal (Fig. 8) enamel surfaces act as dialyzing membranes. Before equilibrium has been reached between the salt concentration on the two sides of the surface cuticle, a great accumulation of fluid is noticed below the membrane which causes expansion, swelling, and tearing of the organic membranes. This latter detrimental action cannot be eliminated by the suppression of CO_2 formation, or by means of pressure. By grinding off the surface before decalcification, that is, by removing the membrane, and thereby preventing the dialysis and at the same time suppressing the CO_2 formation, it has been found that the distortion of the remaining organic elements can be reduced to a minimum (Fig. 3). However, it would be preferable if the dialyzing effect and resulting swelling of the surface membrane could be eliminated without resorting to surface grinding. It is believed that this is partly accomplished by: (1) the use of dilute acid; (2) the slow addition of acid during decalcification, and (3) the protection of enamel by encapsulation in a thin shell of hard celloidin before decalcification (pre-embedding).

Fig. 1.—Human enamel seen from inside after total loss of dentin; oldest dental remains excavated from prehistoric grave in Finland. (From Bonsdorrff, P. von: Odont. Tidskr., **40**: 195-212, 1932, Fig. 10a, p. 203.)

Fig. 2.—Human enamel seen from inside after partial and total loss of dentin; remains after boiling of recently extracted teeth in 50 per cent sulphuric acid for from 20 to 30 minutes.

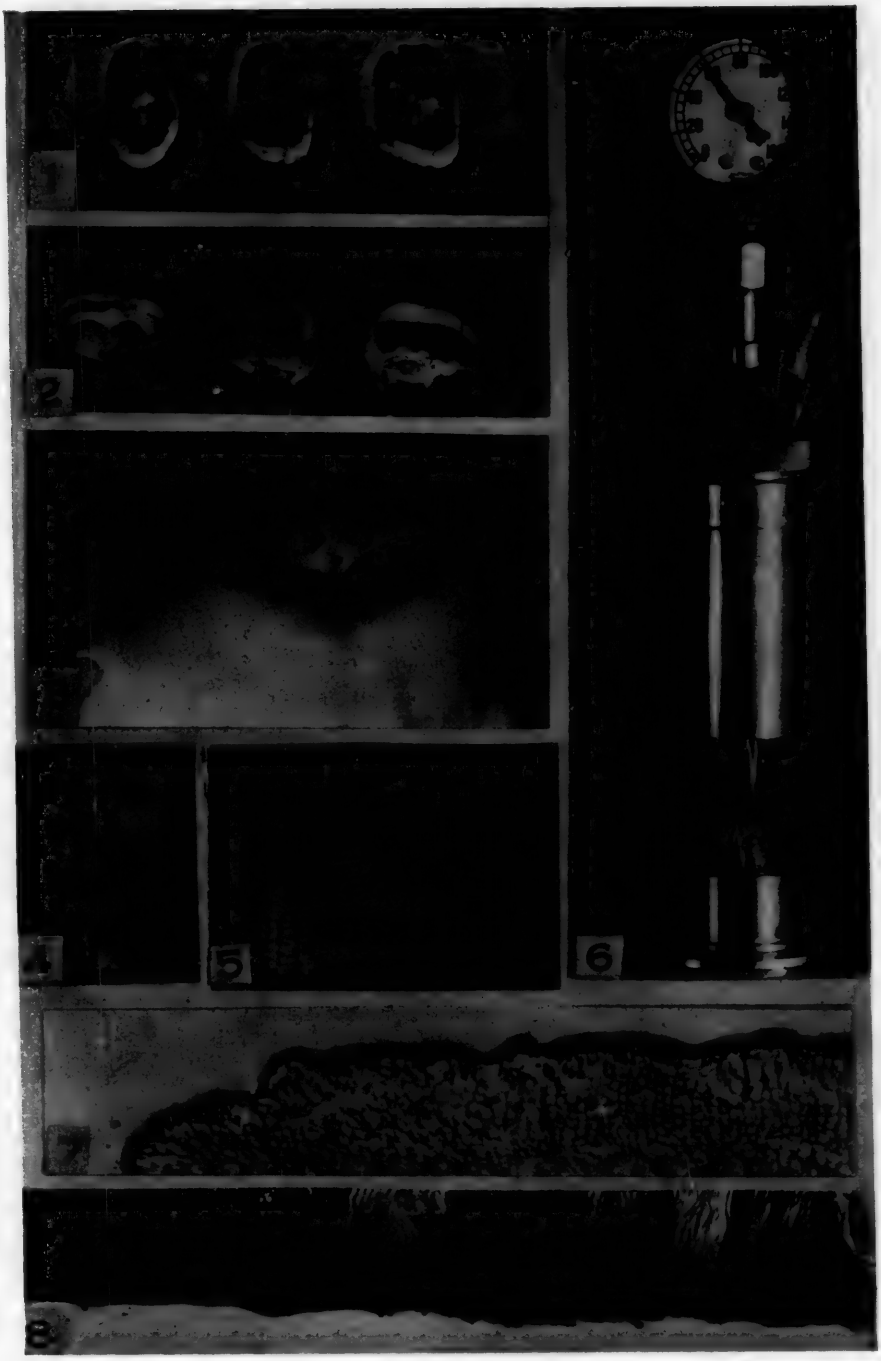
Fig. 3.—Organic matrix of decalcified mature human enamel (external greyish layer). Following removal of most of the surface membrane by grinding, the minerals were completely removed under special precautions described in text. (Specimen immersed in water during the photographing.)

Figs. 4 and 5.—Organic remains dissected from decalcified human molar cusp. External homogenous, "cloudy" enamel matrix (Fig. 4) and internal corrugated organic framework (Fig. 5) successfully demineralized and fixed. (Photograph taken under water.)

Fig. 6.—Miniature pressure decalcifier, showing pressure gauge, air valve, and transparent glass tube which contains tooth suspended in cellodin bag during decalcification.

Fig. 7.—External organic matrix of enamel with surface cuticle. Paraffin section, H & E (200×).

Fig. 8.—Internal, denser, organic framework with basal membrane. Paraffin section, H & E (200×).



(See opposite page for legend.)

In principle, all of these factors tend to slow down the penetration and action of the acid. In that light one now may interpret the advantage of preembedding of the enamel. This addition of a rigid wall around the surface membrane must tend also to slow down the initial penetration of the acid and to prevent the expansion of the surface membrane, in addition to the previously mentioned immobilization of the delicate organic elements once they are freed from their mineral support. The successful use of dilute acid and the slow action of the acid obtained by dropwise decalcification or by a mixture of acid with less dissociating fluids (alcohols or celloidin-alcohol mixtures) may well be explained upon a similar basis. A rapid and forceful CO_2 formation will in the first place be avoided, and the delay in time will permit an equilibrium to be reached on the two sides of the surface membrane.

It has already been noted from Table I that several workers have used methods based upon the simultaneous decalcification and fixation of the enamel. The advantage of this approach has already been discussed in regard to the difficulties inherent in the fixation of intact enamel. However, the choice of acid cannot be limited to considerations of CO_2 formation and protein fixation alone. Low concentrations of chromic acid, trichlor-acetic acid, and others have a satisfactory decalcifying quality with a minimum of CO_2 destruction as well as a precipitating action on the proteins. However, it is also important that the acid prevents swelling of the protein matrix. Schaffer (1902), who has made one of the most extensive studies of the effect of various types of acids and fixatives on hard tissue preparations, has shown that the use of 5 per cent nitric acid in 10 per cent formalin gives the most favorable decalcification and fixation with a minimum of swelling and maximum maintenance of staining property. But this strength of acid cannot be used on enamel unless CO_2 formation is suppressed directly by such involved methods as that of Forshuvud, the essential principle of which has been confirmed but the application of which requires more study.

It would indeed be misleading at this time to give the impression that any specific method is satisfactory for routine use. For experimental purposes the apparatus shown in Fig. 9 has solved some problems. This decalcifier consists of two pressure tanks made out of the acid-resistant methyl-metharcrylate "Lucite" plastic and connected by means of acid-resistant "Inconnel" metal tubes and valves. Installed in an icebox under constant and low temperature, the specimen is placed in individual celloidin bags in the left-hand tank which then is closed and brought under pressure from a compressor placed outside the icebox. Then the right-hand tank is filled with the decalcification agent, which under overpressure is brought into the tank containing the specimen. From then on the specimen is maintained under the same pressure and low temperature all through changes of acid, neutralization and washing. This offers essential advantages over Forshuvud's method in which the release of pressure for the purpose of changing, neutralization, or washing out of the acid tended to ruin whatever initial success could be achieved in the decalcification process. Results obtained by the doube-tank decalcifier indicates that the process of decalcification is a most essential factor involved in the histological preservation of the organic framework of the enamel.

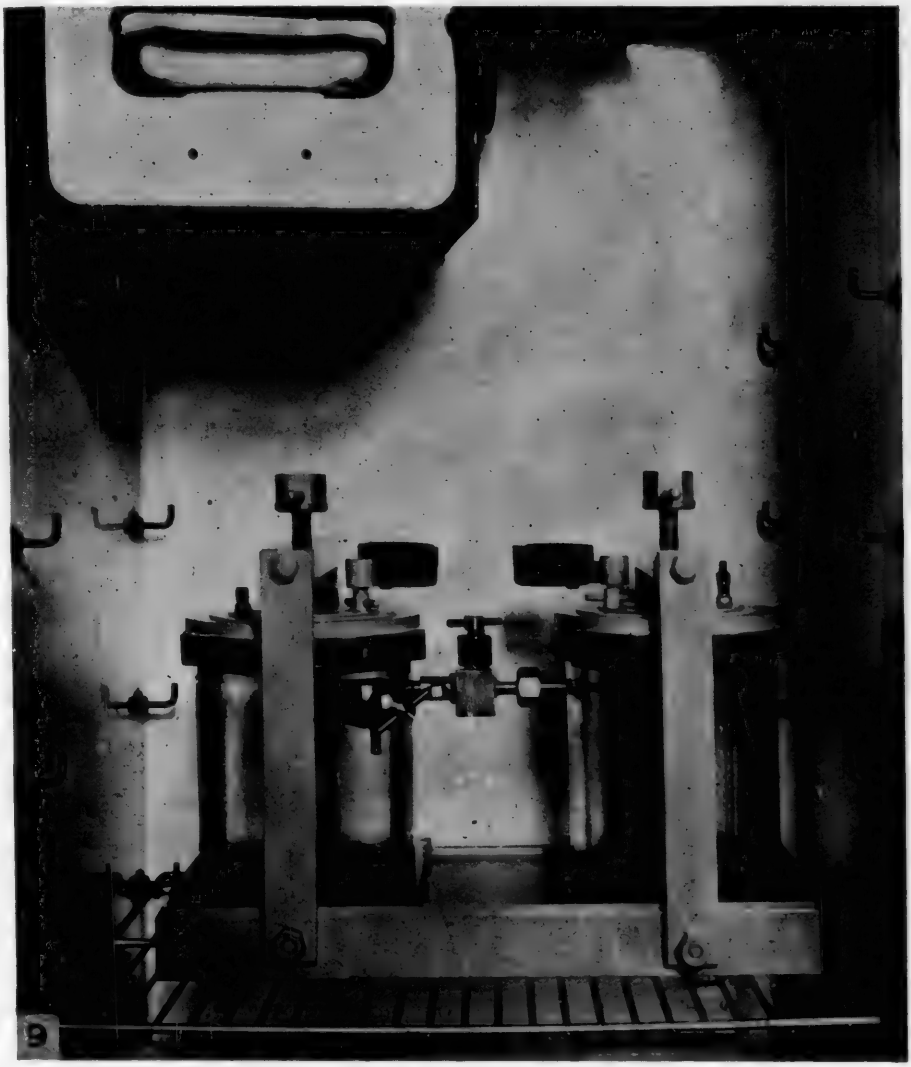
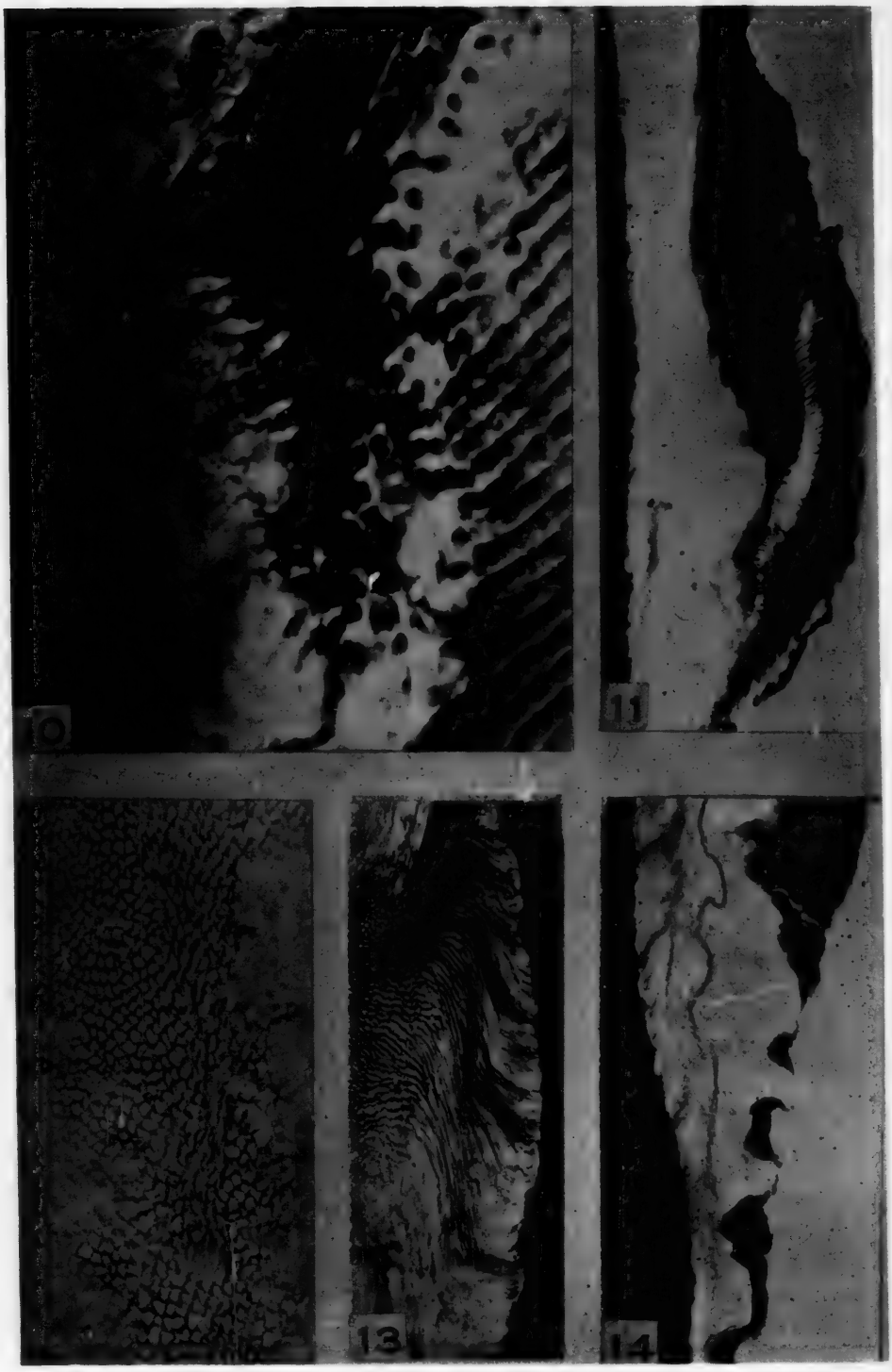


Fig. 9.—Double pressure decalcifier made from Lucite plastic and connected by acid-resistant Inconnel metal tubes and valves.

Even under well-controlled conditions, it should be pointed out that one of the greatest difficulties in testing the value of different histological procedures is the fact that the enamel of different teeth rarely reacts in the same way to identical technical treatment. Indeed, even within the same tooth, there is often evidence of different reactions to the influences of the same chemicals used for histological preparation. Figs. 10 to 14 illustrate various parts of such a tooth which, after uniform exposure to acid under a constant environment, was over-



Figs. 10 to 14.—Decalcified deciduous molar showing underdecalcified (Figs. 10 and 11), well decalcified (Figs. 12 and 13), and overdecalcified (Fig. 14) enamel matrix.

decalcified on one side and underdecalcified on another. This observation of great differences in the organic remains of demineralized enamel is of considerable interest in itself. In concluding this discussion, it primarily serves to show that it is not a simple matter to determine to what extent certain histological results are dependent upon the character of the enamel on the one hand, and upon the chemicals to which the specimen is exposed on the other.

SUMMARY

Since there is no evidence that intact mature enamel lends itself to effective fixation by routine fixation procedures, it is suggested that this problem must be solved by a more rational approach than mere expenditure of fixation time. The importance of fixation and immobilization of the delicate organic elements of the enamel is suggested by the success of histological methods using acids which at the same time act as good protein fixatives by the combination of acids with fixation fluids on the one hand and with embedding mediums on the other.

An analysis of present knowledge suggests that the decalcification process itself is the most critical single step in the successful histological preparation of highly calcified structures. The isolation of the intact organic framework, in its normal relationship to its surroundings, is benefited by the suppression of forceful CO_2 formation, and by the prevention of a rapid dialyzing action in the presence of organic surface membranes.

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THE LOCATION OF THE ZONE OF DISINTEGRATING EPITHELIAL ATTACHMENT CUTICLE

Relation to the Cemento-enamel Junction and to the Outer Border of the Periodontal Fibers on Some Tooth Specimens

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THE epithelial attachment cuticle *(eac)* is a cuticular membrane which underlies the epithelial attachment *(ea)* and attaches the innermost cells of this tissue to the tooth, whether it is located on the enamel or on the cementum. This cuticle is probably produced or laid down by the cells of the epithelial attachment.[1-4]

Wherever it is located at any time the outer border of the epithelial attachment cuticle is undergoing more or less breaking down or disintegration and as a result stains heavier than the remaining uninjured portion. This heavier staining line or strip may be demonstrated readily on extracted tooth specimens. Bass[5] has called it the zone of disintegrating epithelial attachment cuticle *(zdeac)*.

The outer border of the epithelial attachment comes right up to the zone of disintegrating epithelial attachment cuticle but does not overlap it. Therefore this line accurately indicates the location of the outer border of the epithelial attachment at any place around a given tooth specimen. It also accurately indicates the exact extent to which the epithelial attachment cuticle has been destroyed, and is now being destroyed, at any place.

The purpose of this paper is to show the location of the zone for disintegrating epithelial attachment cuticle, the cemento-enamel junction *(cej)*, and the outer border of the periodontal fibers *(pf)* on a number of selected tooth specimens.

MATERIAL AND METHODS

Specimens used for this purpose were selected from a large miscellaneous collection of formalin-preserved extracted teeth obtained from a number of different sources. An attempt was made to select representative specimens and not specimens showing unusual or rare conditions.

The method previously described[5] was employed in preparation and examination of specimens. Any excessive adhering soft tissue was trimmed away. Undesirable (for this purpose) soft bacterial and cellular material was removed

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by brushing with a toothbrush. Vigorous brushing does not remove the zone of disintegrating epithelial attachment cuticle (it does remove some of the loose pieces at the outer border of the zone of disintegrating epithelial attachment cuticle), the epithelial attachment cuticle or the periodontal fibers. Most, if not all, adhering epithelial cells are removed by the brushing. The specimen was stained with crystal violet (0.5 per cent in H_2O) one to three minutes and again brushed off gently.

Drawings were made with the aid of an Abbé type camera lucida (Zeiss) at approximately ×112 original magnification. Outlines were accurately traced. The zone of disintegrating epithelial attachment cuticle blends or shades into the uninjured epithelial attachment cuticle; therefore the distinction between them is not usually as sharp as these pictures indicate. The outer side of the zone of disintegrating epithelial attachment cuticle, which consists of breaking up cuticular material,[5] is a more or less irregular line when viewed under considerable magnification. Notwithstanding these limitations, the drawings correctly indicate the general outline of the disintegrating zone.

The outline of the outer border of the periodontal fibers on the tooth was accurately drawn and the fibers at the border indicated. Fibers and periodontal tissue farther down on the root are suggested only by a few miscellaneous lines. An effort was made to trace and indicate accurately the outline of the root itself.

Calculus, bacterial film, and other material on the specimen above the zone of disintegrating epithelial attachment cuticle are not indicated (except in Fig. 1); neither are cavities nor other abnormalities.

Whenever the cemento-enamel junction was obscured by overlying calculus or other material, this material was removed to permit accurate tracing of the junction line (the *cej*) between the enamel and the cementum.

When a tooth is extracted, most of the tissue of the epithelial attachment is torn from it and remains in the mouth. The epithelial attachment cuticle, whether on the enamel or on the cementum, always remains firmly attached to the tooth. Usually a varying number of the epithelial attachment cells remain attached to the cuticle, and these may be recognized and identified by appropriate technic.[5] A convenient quick way is to stain (H & E or other stain) an extracted tooth specimen and examine it with suitable magnification under incident light. Higher magnification and transmitted light may be used to examine specimens of the epithelial attachment cuticle and the cells thereon, removed from the enamel with the aid of acid (10 per cent HCl in H_2O). Paraffin or celloidin sections of decalcified teeth may be made for similar examination, including the epithelial attachment cuticle and any adhering cells on the cementum.

When a tooth is extracted, most of the periodontal fibers are torn, the dental end remaining firmly attached to the cementum. By suitable technic, the location of the outer border of these fibers on the tooth may be recognized satisfactorily. In this way one may observe exactly to what extent, from the cemento-enamel junction apexward, the fibers have been destroyed and have been replaced by epithelial attachment.

CHANGES THAT OCCUR IN THE LOCATION OF THE EPITHELIAL ATTACHMENT, EPITHELIAL ATTACHMENT CUTICLE, ZONE OF DISINTEGRATING EPITHELIAL ATTACHMENT CUTICLE, AND PERIODONTAL FIBERS

On a normal tooth, stabilized in normal occlusion, the epithelial attachment extends from the cemento-enamel junction occlusalward to the zone of disintegrating epithelial attachment cuticle. The underlying epithelial attachment cuticle extends from the cemento-enamel junction occlusalward to, and including, the zone of disintegrating epithelial attachment cuticle (Figs. 1, 2). Therefore the epithelial attachment, together with its underlying cuticle, is normally located on the anatomical crown.

Normally periodontal fibers cover the entire root from the cemento-enamel junction to the apex (Figs. 1, 2). An important function of the cementum is to imbed and anchor the dental ends of the different groups of these fibers to the tooth. As the outermost fibers break down or disintegrate and are removed from the surface of the cementum (in the usual course of the disease process, periodontoclasia), the epithelial attachment grows apexward, crossing over the cemento-enamel junction, and covering the area of cementum from which the periodontal fibers have been removed. At such a place the epithelial attachment is located partly on the enamel above, and partly on the cementum below the cemento-enamel junction (Figs. 3, 4). The occurrence of this condition frequently has been noted by others[6, 7, 8] without, however, emphasizing its true pathological significance, except for Goldman.[6]

As the breaking down and removal of the periodontal fibers progresses further, the epithelial attachment grows farther apexward over the cementum from which the fibers have been removed. Usually, at this stage, the zone of disintegrating epithelial attachment cuticle and the outer border of the epithelial attachment recede at about the same rate as the inner border of the attachment advances along the tooth. In due course, the full width of the epithelial attachment and the zone of disintegrating epithelial attachment cuticle may be located entirely below the cemento-enamel junction at a given place around a tooth (Figs. 5-10). As the process continues, the epithelial attachment progressively moves apexward.

As the attachment grows apexward, simultaneously cuticle is produced beneath it. This attaches the new epithelial cells to the cementum. Therefore the epithelial attachment and its underlying cuticle extend from the zone of disintegrating epithelial attachment cuticle at any given place to the outer border of the remaining intact periodontal fibers.

COMMENT

The conditions indicated in these illustrations, of variation (sometimes great, as in Figs. 4, 7, 8), in the receding of the soft tissues attached to the tooth at different locations around it, are more in keeping with a local pathological process (periodontoclasia) and not with a theoretical physiological process of "continuous passive eruption."

Fig. 1.

Fig. 2.

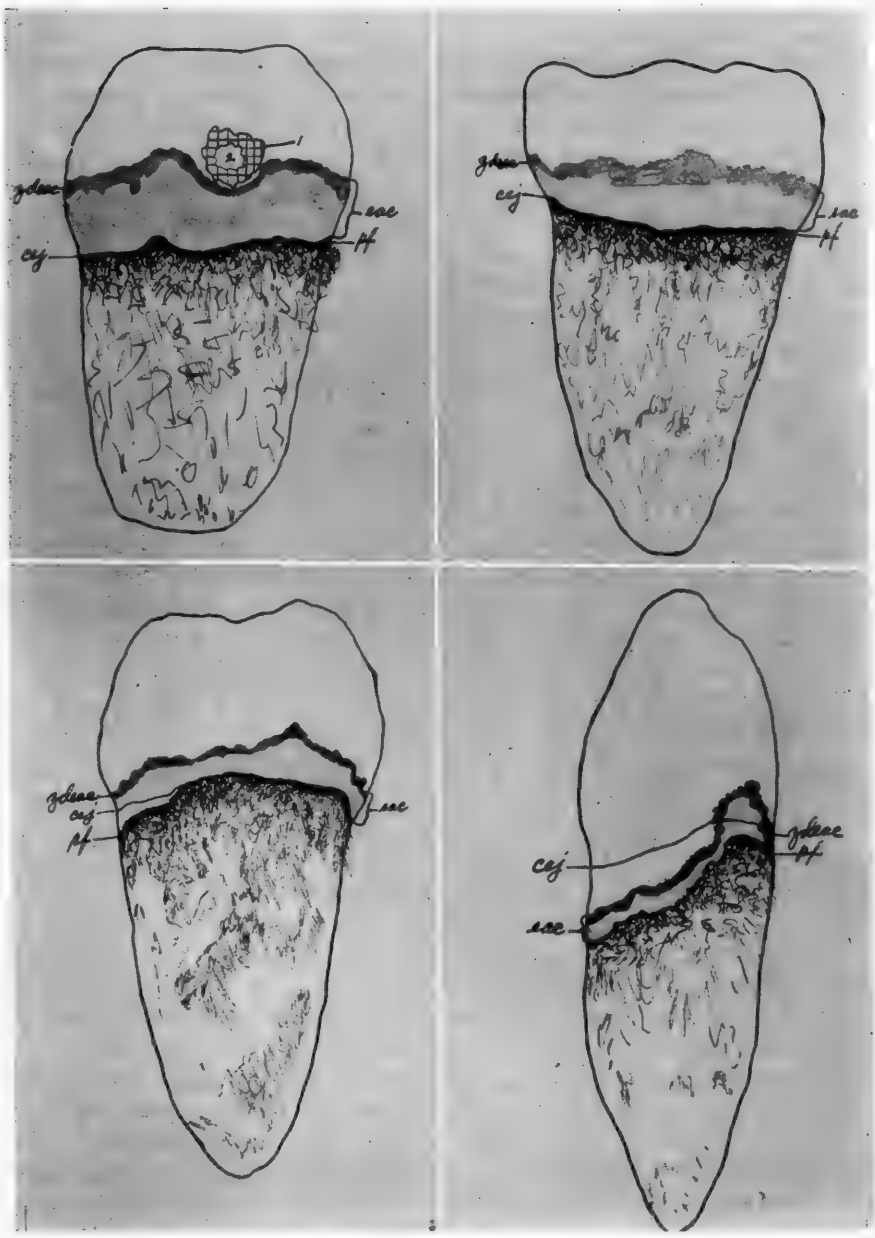

Fig. 3.

Fig. 4.

Fig. 1.—Young tooth. *Zdeac* entirely on the anatomical crown; note how it dips below the disc of bacterial film I, which surrounds the contact plane 2; *pf* come to the *cej* all the way around the tooth.

Fig. 2.—*Zdeac* entirely on the anatomical crown; *pf* come to the *cej* all the way around the tooth.

Fig. 3.—*Zdeac* entirely on enamel above *cej;* at left *eac* partly above and partly below *cej; eac* narrower than in Figs. 1 and 2; *pf* come to *cej* except at left where they have been destroyed to below that line.

Fig. 4.—*Zdeac* mostly below *cej,* but on the right still remains above; *pf* well below the *cej,* much lower on the left than on the right.

Fig. 5. Fig. 6.

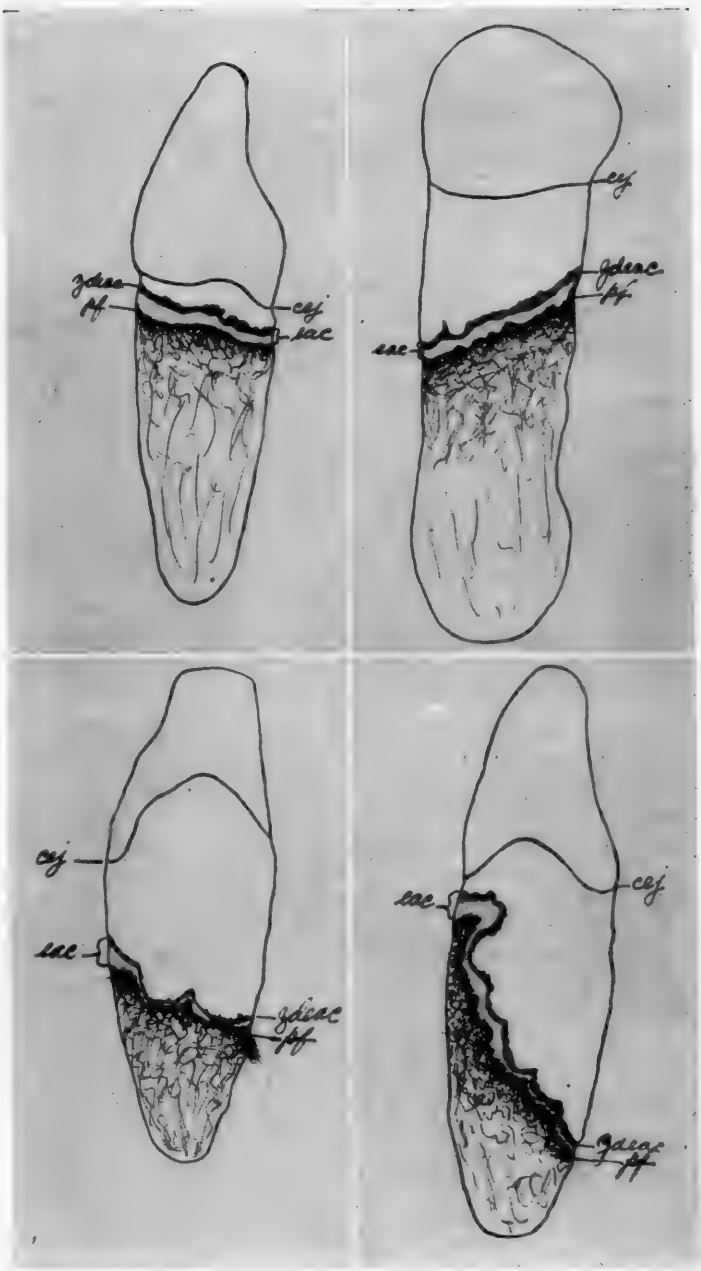

Fig. 7. Fig. 8.

Fig. 5.—Entire *eac* including *zdeac* well below *cej*; note fairly uniform width of the *eac* between the *zdeac* and the *pf*. Note the distance to which the *pf* have been destroyed from the *cej* where they are normally located.

Fig. 6.—Old tooth with worn down crown; enlarged and elongated root—hypercementosis; *zdeac* and *pf* far below *cej*.

Fig. 7.—Old tooth incisal edge worn down; *zdeac* and *pf* far below *cej*. Visualize the *pf* up at the *cej* where they are normally and one can imagine the serious impairment of function which must result from so great a loss of the supporting structures around the tooth.

Fig. 8.—*Zdeac* and *pf* relatively high towards the labial, running obliquely across and downward to the lower fourth of the root on the lingual. This is not an unusual picture for the advanced stage of periodontoclasia involving lower incisors and canines.

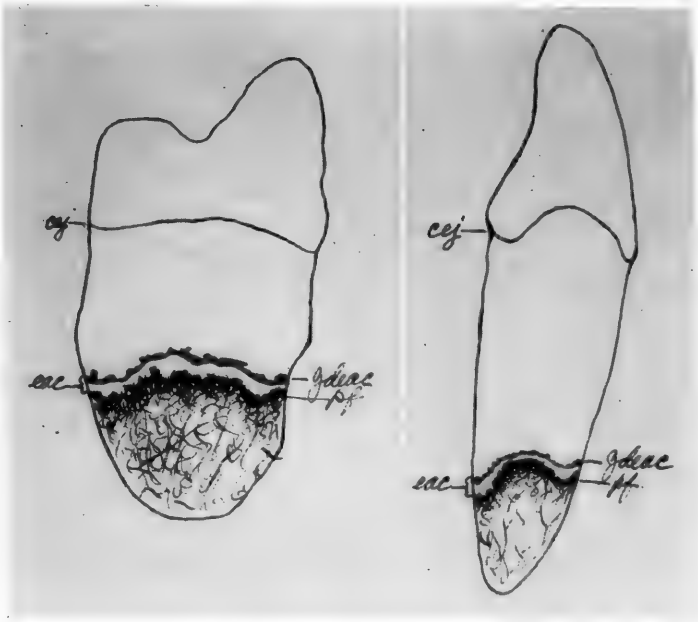

Fig. 9. Fig. 10.

Fig. 9—Old tooth, worn down crown; *zdeac* and *pf* far below *cej*. Tooth had less than one-third of its normal support.

Fig. 10.—*Zdeac* far from its normal location on the anatomical crown; *pf* far below *cej* to which they normally extend.

SUMMARY

The location of the zone of disintegrating epithelial attachment cuticle in relation to the cemento-enamel junction and to the outer border of the periodontal fibers on some extracted tooth specimens has been indicated by means of camera lucida drawings. The explanation of "continuous passive eruption" as a physiological process is questioned.

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CHEMICAL ANALYSIS OF TEETH SHOWING UNUSUAL WEAR

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THE constancy of composition of tooth substance is an old and well-established fact. In some abnormalities, such as hereditary opalescent dentin (dentinogenesis imperfecta), the organic to inorganic ratio is changed but, as far as is known, without exception, the mineral phase in any solid calcium phosphate of the body has been found to be an apatite with fairly constant calcium and phosphorus percentages. The present study re-emphasizes the constancy of tooth composition even in teeth which might well have been supposed to differ in some detectable way.

Teeth from a full mouth extraction were examined in this study. All teeth were present except the maxillary right first molar. It was noted that all the teeth on the left side of both upper and lower jaws showed marked attrition so that they were without cusps, while those on the right all had normal contour (Figs. 1, 2, and 3).* In seeking an explanation of the unilateral wearing away of tooth structure, the possibility was raised that differences in chemical constitution were responsible. In these analyses, determinations were made on separated enamel and dentin for the following constituents: organic matter, total inorganic matter, calcium, phosphorus, and magnesium. Calcium/phosphorus, and magnesium. Calcium/phosphorus and calcium/magnesium ratios were computed. Microhardness readings were taken in the dentin on sections cut from the whole teeth. A similar study of an asymmetry of tooth composition has been published by LeFevre and Hodge[1] on whole teeth in which differences in hardness and in inorganic matter were reported.

METHOD

The teeth from the full mouth extraction of one patient (B. E. R.) were obtained from Dr. J. Frank Hall, Dental College of Virginia. The teeth were cleaned, and the small amount of carious material present was removed. Microhardness was determined by the Bierbaum microcharacter on slabs cut from the whole teeth.[2] Following this process, the enamel and dentin were separated by the Manly and Hodge flotation process.[3] Organic matter was extracted by boiling in 3 per cent potassium hydroxide in ethylene glycol.[4] Calcium determinations were made on the inorganic residue following the method of LeFevre and Nicholson[5]; samples transformed into calcium oxalate were titrated against ceric sulfate. Phosphorus was precipitated as phosphomolybdate according to Holtz.[6] Magnesium was precipitated with 8-hydroxyquinoline according to Greenberg, Anderson, and Tufts[7] and Greenberg and Mackey.[8]

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*The patient had visited the clinic for the extraction of the two teeth not shown in the photographs a few weeks before these pictures were taken.

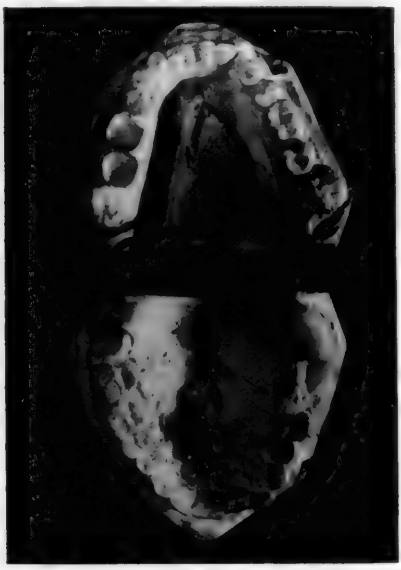

Fig. 1.—Cast of upper and lower teeth. The occlusal surfaces of the teeth on the left sides of both jaws are flat or even cupped. The cuspal formation of the teeth on the right sides of the jaws appears to be normal.

Fig. 2.

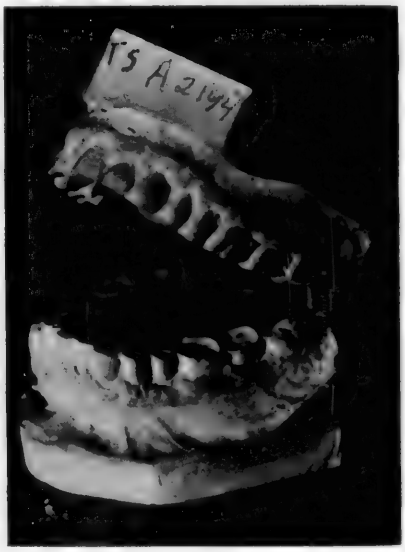

Fig. 3.

Fig. 2.—Cast of the upper and lower teeth. This view of the right sides of the jaws shows that the cuspal contours change abruptly to cupped surfaces on the teeth of the left sides.

Fig. 3.—Cast of the upper and lower teeth. The ridges of enamel surrounding the occlusal surfaces are raised about the level of the dentin giving the appearance of shallow cups resembling Petri dishes to the first and second lower molars.

DATA

The detailed chemical data are presented in Table I. Percentage of organic matter is listed as per cent dry weight. Calcium, phosphorus, and magnesium percentages are calculated as per cent of inorganic residue. Ratios are determined between calcium and phosphorus and calcium and magnesium percentages. Table II is a summary of Table I.

DISCUSSION

Fig. 1 is a photograph of the teeth of patient B. E. R. The cuspless teeth may be seen on the left sides of the jaws. The patient was a white man, 44 years old. He was in good general health. Ten years earlier he had had treatments for periodontal disease, and at the time of extraction, that condition was well advanced. The teeth were practically free from caries. All were present except the maxillary right first molar. There was extensive loss of alveolar bone about all teeth; a clinical impression was that the loss may have been more extensive on the left side corresponding to the attrition of the teeth on that side. The remaining alveolar bone seemed to be of good quality. There is no available information concerning hereditary, anatomical, or physical causes for the attrition of the left cusps.

Microhardness values for the dentin are given in the first column of Table I. The average hardness for the teeth on the right is almost numerically identical with that for the teeth on the left (right, 109, left, 107). Considering the teeth in pairs according to location, right vs. left, in 6 cases the value on the right is higher, in 3 cases the value on the left is higher, and in 7 cases there is no difference.

The organic percentage is higher for the teeth on the left in both enamel and dentin fractions. The averages (Table II) verify this trend. Organic matter in enamel is 4.7 per cent for the left compared with 3.2 per cent for the right; in dentin there is the same picture, left, 18.5 per cent, right, 17.6 per cent. Neither of these differences is outside the range included by the standard deviation, but nevertheless, the possibility remains that there may be a greater amount of organic matter in the teeth on the left. This fact would be in line with the assumption that the flat teeth were approaching the composition of bone.

There is very little difference in the calcium content of the glycol ash, right vs. left; in fact, the averages are almost identical (enamel, right, 36.3 per cent, left, 36.4 per cent; dentin, right 33.4 per cent, left, 33.7 per cent).

There is no appreciable difference between the phosphorus content of enamel and dentin between the normal and cuspless teeth. The averages are for enamel, right, 17.5 per cent, left, 17.5 per cent, and for dentin, right, 16.3 per cent, and left, 15.8 per cent.

The calcium/phosphorus ratios show no significant differences; their averages lie close together within the normal range. Magnesium percentages show no definite trend. Calcium/magnesium ratios appear normal.

In conclusion, in the presence of marked loss of tooth substance of all the teeth on the left sides of the jaws, there appears to be no marked variation in

TABLE I

COMPOSITION OF TEETH OF B. E. R.

TOOTH POSITION	TOOTH NUMBER	MICRO-HARDNESS DENTIN	ORGANIC MATTER* %		INORGANIC MATTER* %		CALCIUM† %		PHOSPHORUS† %		MAGNESIUM† %		CALCIUM/PHOSPHORUS		CALCIUM/MAGNESIUM	
			ENAMEL	DENTIN	ENAMEL	DENTIN	ENAMEL	DENTIN	ENAMEL	DENTIN	ENAMEL	DENTIN	ENAMEL	DENTIN	ENAMEL	DENTIN
$\frac{1}{}$|	479	102	1.4	20.9	98.6	79.1	36.2	31.4		15.3		1.08		2.05		29.1
|$\frac{1}{}$	477	141	2.0	18.0	98.0	82.0	37.0	34.6	17.5	16.2		0.90	2.11	2.14		38.4
$\frac{1}{}$|	475	120	1.4	24.2	98.6	75.8	36.8	33.1	20.7	15.6		1.45	1.78	2.12		22.8
|$\frac{1}{}$	480	87	2.4	19.0	97.6	81.0	36.7	35.6	16.1	16.6		1.50	2.28	2.14		23.8
$\frac{2}{}$|	456	86	4.5	17.5	95.5	82.5	34.7	32.4	17.8	16.5		1.25	1.95	1.96		26.0
|$\frac{2}{}$	481	84	9.8	15.0	90.2	85.0	36.9	30.7		16.7		1.21		1.84		25.4
$\frac{2}{}$|	464	62		25.4		74.6	37.3	35.0	17.5			1.04	2.13			33.7
|$\frac{2}{}$	454	64	11.7	20.5	88.3	79.5	35.9	34.0	18.6			1.36	1.93			25.0
$\frac{3}{}$|	478	139		14.7		85.3	36.8	34.4	19.3	17.0		0.96	1.91	2.02		35.9
|$\frac{3}{}$	471	115	3.8	18.2	96.2	81.8	36.8	34.1	17.1	16.4	0.65	1.28	2.15	2.08	56.7	26.6
$\frac{3}{}$|	468	83	6.1	18.0	93.9	82.0	36.5	33.1	16.8	16.2		1.20	2.17	2.03		27.6
|$\frac{3}{}$	470	112	6.6	25.0	93.4	75.0	35.6	33.2		15.3		0.84		2.17		39.5
$\frac{4}{}$|	462	118		13.5		85.6				15.3		1.32				30.6
|$\frac{4}{}$	459	122	lost													

$\frac{4}{\|}$	473	118	6.6		93.4		36.9		17.0				217			
$\frac{\|}{4}$	469	125	4.1	14.1	95.9	85.9	36.4	33.9	17.0			1.41	2.14			24.0
$\frac{5}{\|}$	457	85	2.3	16.7	97.7	83.3	36.2	32.3	16.0		0.16	1.14	2.26		226.2	28.3
$\frac{\|}{5}$	483	109		24.2	97.2	75.8	38.0	32.8	20.3	14.3		1.10	1.87	2.29		29.8
$\frac{5}{\|}$	463	119	0.7	12.1	99.3	87.9	35.6	35.7	17.4	16.8		1.10	2.05	2.12		32.4
$\frac{\|}{5}$	476	99	2.4	17.5	97.6	82.5	36.0	33.6	15.5	16.0		1.18	2.32*	2.10		28.5
$\frac{6}{\|}$																
$\frac{\|}{6}$	466	119	2.2	16.3	97.8	83.7	35.8	34.0	17.5	16.3	1.16	1.21	2.05	2.09	30.9	28.1
$\frac{6}{\|}$	474	105	1.3	16.9	98.7	83.1	36.6	32.6	15.9	16.3	0.64	1.02	2.30	2.00	57.3	32.0
$\frac{\|}{6}$	461	100	11.0		89.0		36.9	34.4		15.3		1.25		2.25		27.5
$\frac{7}{\|}$	458	113	2.5	19.9	97.5	80.1	36.9			16.2		1.23				
$\frac{\|}{7}$	455	84		16.4		83.6	35.5	34.2	18.4	18.6		1.31	1.93	1.84		26.1
$\frac{7}{\|}$	467	135	0.9	14.4	99.1	85.6	35.2		16.7	19.0	0.63	0.76	2.11		55.8	31.8
$\frac{\|}{7}$	482	135	0.9	19.2	99.1	80.8	37.1	31.7	18.6	14.8		1.28	1.99	2.09		24.8
$\frac{8}{\|}$	472	120	7.7	16.9	93.3	83.1	37.0	33.7	17.7	15.7	0.52	1.16	2.09	2.15	71.2	29.0
$\frac{\|}{8}$	465	89	2.1	16.5	97.9	83.5	35.7	34.8	17.4	15.5		0.98	2.05	2.25		35.6
$\frac{8}{\|}$	460	136	3.0	15.0	97.0	85.0	35.2		17.8	15.5	0.80	1.64	1.98		44.0	
$\frac{\|}{8}$	484	121	2.1	19.5	97.9	80.5		34.0		14.0	0.60	0.98				34.7

*The percentages of organic matter and inorganic matter are expressed as percentages of dry weight.
†The percentages of calcium, phosphorus, and magnesium are expressed as percentages of ash weight.

TABLE II
COMPOSITION OF TEETH FROM RIGHT AND LEFT SIDES OF MOUTH

	ENAMEL			DENTIN		
	RIGHT	LEFT	AVERAGE	RIGHT	LEFT	AVERAGE
Microhardness				109	107	108
Organic matter %	3.2	4.7	4.1	17.6	18.5	18.1
Inorganic matter %	96.8	95.3	95.9	82.4	81.5	81.9
Calcium %	36.3	36.4	36.4	33.4	33.7	33.6
Phosphorus %	17.5	17.6	17.5	16.3	15.8	16.1
Magnesium %	0.55	0.8	0.64	1.17	1.18	1.18
Calcium/Phosphorus	2.08	2.06	2.06	2.06	2.11	2.08
Calcium/Magnesium	90.9	43.8	77.4	29.9	29.2	29.5

chemical composition although the slightly greater organic content of the cuspless teeth may be evidence of a significant decrease in resistance to attrition. The difference in contour presumably has a physical, anatomical, or functional basis. There may have been continued attrition of the teeth of the left side by excessive trauma such as chewing exclusively on one side of the mouth.

SUMMARY

Teeth from a full mouth extraction were analyzed for organic matter, calcium, phosphorus, and magnesium. The teeth on the right side of the jaws all had normal contours while those on the left were cuspless. Chemical analysis showed no large differences in the composition of the teeth on the two sides of the mouth.

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RADIOACTIVE PHOSPHATE PERMEABILITY IN DENTIN FOLLOWING THE USE OF MEDICAMENTS

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INTRODUCTION

DENTIN has been shown to be permeable to dyes,[1-4] to salts such as silver nitrate and sodium fluoride,[5-7] to radioactive salts,[8, 9] and even to bacteria.[10]

Wasserman, Blayney, Groezinger, and DeWitt[8] maintained that in teeth in which pulps were removed, dentin absorbed P_{32} from the saliva, and when teeth were sealed from the saliva by means of cemented gold crowns, the dentin absorbed P_{32} from the pulp and periodontal tissues. They further claimed that teeth with pulps removed take up P_{32} at a lesser rate than intact teeth. Others [11, 12] have shown that dentin permeability to P_{32} varies with different morphological and pathological varieties.

Lefkowitz[1] maintained that the diffusion of dye substance through the dentin follows a definite pattern dependent on the dentinal lymph flow. Zander and Smith,[6] on the other hand, indicated that the penetration of silver nitrate solution in dentin is not associated necessarily with the flow of a tissue fluid.

Zander[13] and Zander and Pejko[14] studied the penetration of dental silicate cements (alone, and as influenced by zinc oxyphosphate cement and cavity varnish) and their effects on dentin permeability by measuring clinical responses to the electric pulp tester and by examining histological sections of pulp tissue.

Lukomski[15] claimed, on the basis of histological studies, that there is marked alteration in the permeability of dental tissues that have been exposed to concentrated fluoride solutions, and that the permeability of fluoride treated tooth structures to silver nitrate is of greatly reduced magnitude.

Volker and Bibby,[16] in discussing the action of fluorine in limiting dental caries, pointed out that fluorine might increase tooth resistance by its metabolic effect on the enamel and dentin.

This study deals with the effect of various medicaments commonly used in dental practice on the permeability of dentin to radioactive phosphorus in the teeth of dogs.

MATERIAL AND METHODS

Adult dogs were prepared for operation after being completely anesthetized with Nembutal (pentobarbital sodium) by intraperitoneal injection of 1 gram for every 5 pounds of body weight. Two to three cavity preparations (depending on the size of the individual teeth) were made on the mesial or the distal surface of canine teeth. Incisor teeth were extracted where they obstructed access to mesial surfaces.

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Phenol, phenol followed by alcohol, fluorine, silver nitrate followed by eugenol, cavity varnish, and zinc oxyphosphate cement were applied to the inner surface of each respective cavity preparation except in those animals designated to be the control group. Following this, equal volumes of P_{32} solution of Na_2HPO_4 (equivalent to 30μ) were placed in each preparation and then followed by a small cotton pellet. The cavity preparations were then sealed with silver amalgam.

Animals were killed after forty-five hours. Four canine teeth were removed from each and sectioned on an abrasive wheel to include all cavity preparations. The teeth were further ground on an India stone to a thickness of from 100 to 150 microns. Autographs were prepared by sections being placed on ultra-speed dental x-ray film and by their being sealed between two glass plates for thirty minutes.

OBSERVATIONS

Both mesial and distal surfaces of twenty-four canine teeth in dogs were used in this experiment.

In Fig. 1, from a dog's lower left canine, the three cavity preparations are, from crown to apex, control, oxyphosphate cement, and 10 per cent phenol in alcohol. Fig. 2 is the corresponding radio-autograph. Where the cement was in contact with the dentin, no P_{32} penetrated into the dentin; this area directly under the base of the cavity preparation is in sharp contrast to the cavity walls where no oxyphosphate cement was placed. The phenol-treated cavity shows greater penetration than does the control cavity.

In Fig. 3, from a section of a dog's lower right canine, cavity preparations were, from crown to apex, control, cavity varnish, and silver nitrate followed by eugenol. Fig. 4 is the corresponding radio-autograph. The cavity preparation treated with cavity varnish shows greater penetration of P_{32} than does that of the control, and the silver nitrate shows still further penetration than the cavity varnish.

Fig. 5 is a photograph of a section of a dog's upper right canine. The preparations from crown to apex, on the labial surface, were fluorine (saturated aqueous solution) and silver nitrate followed by eugenol. On the lingual surface, the preparations were treated with 10 per cent phenol and 10 per cent phenol followed by 70 per cent alcohol. Fig. 6 is the corresponding radio-autograph. Plain 10 per cent phenol was followed by the greatest penetration of P_{32} into dentin. Phenol followed by alcohol did not result in quite so much penetration. The penetration observed after application of fluorine was greater than that observed when silver nitrate was followed by eugenol, but it was not quite so much as that observed when phenol was followed by alcohol.

The resultant autographs of all experimental material indicate that while the zinc oxyphosphate cement was completely impervious to the penetration of P_{32}, all other medicaments were followed by greater penetration than would normally have occurred without the use of any medicaments. P_{32} penetration of the dentin was highest in the phenol-treated cavity, less, in the order indicated, in phenol followed by alcohol, fluorine, silver nitrate followed by eugenol, and cavity varnish.

Figs. 1-6.

DISCUSSION

Clinically, it would be desirable to decrease the permeability of the dentin in order to diminish pulpal reaction to external stimuli and to lessen the possibility of bacterial invasion and the opportunity for recurrent decay. Medicaments such as silver nitrate followed by eugenol, phenol, phenol followed by alcohol, and fluorine have been used not only as sterilizing agents,[17] but with the expectancy that they coagulate the dentinal fluid and dentinal lymph,[18]

thereby sealing off the exposed dentinal tubules and decreasing the permeability of dentin.

From theoretical considerations there is no logical basis for the assumption that coagulation and consequent death of living protoplasm in the dentinal tubules will decrease permeability. Considering the behavior of living cells such as the amoeba and red blood cells, the opposite appears to be true. Cell membranes which are highly selective during life acquire unlimited permeability upon death. Osterhout[3] stated, "Death is accompanied by an increase in permeability . . . It is a matter of common observation that cells may resist the penetration of certain dyes as long as they are alive, but absorb them readily as soon as they are killed." Others have shown that after pulp necrosis (and consequent death of the Tomes' dentinal fibers), experimental dyes will diffuse through the dentin profusely,[2] and the tooth of a dog will take up dyes in the same manner as "a sponge takes up water."[19] The penetration of radioactive phosphorus and experiments with dyes indicate that the commonly used medicaments do not have the pharmacological action expected of them. On the contrary, they appear to have the opposite action in actually increasing the permeability of the dentin instead of decreasing it. The only justification in using these medicaments would be for their germicidal properties, but in the light of the work of Besic[10] in which he showed that bacteria die out when sealed in cavities under fillings, it might be advisable to avoid using these powerful caustics prior to inserting filling materials.

CONCLUSIONS

1. The effect of commonly used medicaments on the permeability of dentin has been studied.

2. Oxyphosphate cement base proved to be completely impervious to the penetration of a solution of P_{32}. In all other cases penetration was greater than in the corresponding control cavity.

3. Considering the effect of the commonly used medicaments on the vital protoplasm in the dentinal tubules, it might be advisable to discard their use in favor of less caustic medicaments.

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THE DEVELOPMENT AND MORPHOLOGY OF PHYSIOLOGICAL SECONDARY DENTIN

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INTRODUCTION

IN A previous report, Bevelander and Benzer[1] differentiated physiological secondary dentin and adventitious secondary dentin and described the incidence and the various morphological types of adventitious secondary dentin that are deposited in human teeth. A continuation of this study reveals additional information regarding the morphology and the development of the physiological secondary dentin, that is, dentin which is not formed as a result of stimuli.

Reich[2, 3] reported on this subject and described a layer of dentin which he maintained is a new physiological development and not a tissue of a senile character. This layer he called dentin of the "functional period." Kanamori,[4] in a histological study of ovarian dermoid cysts, presented, as part of his report, photographs of sectioned teeth which developed in the cysts. All the elements of normal teeth were shown to be present: enamel, dentin, cementum, and pulp, plus physiological secondary dentin, sharply demarcated from the primary dentin. Fish[5, 6] maintained that regular secondary dentin under the occlusal surface of the molars (accelerated growth line) is brought about by irritation resulting from caries. Kronfeld[7] stated that most intact teeth in older individuals show dentin of an irregular nature and the probable explanation for the appearance of this dentin is pulp atrophy. Schour and Massler[8] reported on the growth of enamel and primary dentin, but did not include physiological secondary dentin as part of the growth pattern. Gurley and Van Huysen[9, 10] from their experiments on dogs maintained that the formation of adventitious secondary dentin can be induced by the use of various filling materials which act as stimuli on the receptive pulp.

This report is concerned with the following aspects of the growth and maturation of dentin: (1) The study of the chronological development of physiological secondary dentin; (2) observations concerning the morphology of physiological secondary dentin; (3) the correlation between the function and appearance of the "functional" secondary dentin; and (4) the differentiation between physiological secondary dentin, primary dentin, and adventitious secondary dentin.

MATERIALS

Permanent erupted and nonerupted single-rooted and multirooted human teeth of various age levels were examined. These teeth were extracted for prosthetic, orthodontic, or surgical reasons. The majority of the teeth were of normal morphology. A few were extracted because caries had extended to the pulp tissue. The specimens were ground on a rotary abrasive wheel which was continually lubricated with a stream of cold water. Specimens were cut

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on the long axis of the tooth in such a way as to give the longest possible section and to show the complete root canal and pulp chamber. For ideal observation, the sections were mounted in water and studied with transmitted and reflected light with a stereoscopic and a regular compound microscope. In all, 142 teeth were examined. This group included twenty unerupted multirooted teeth, eighteen unerupted single-rooted teeth, fifty-one erupted multirooted, and forty-nine single-rooted teeth, as well as three over-retained deciduous canines and one tooth obtained from an ovarian cyst.

DEVELOPMENT OF PHYSIOLOGICAL SECONDARY DENTIN

In an attempt to ascertain the relation between the appearance of physiological secondary dentin and the chronological age of normal teeth, a series of 142 teeth were examined, as previously explained.

Multirooted Teeth.—A series of fifty-one erupted molars obtained from patients ranging from 8 to 65 years of age reveals that physiological secondary dentin is completely developed a few years after eruption and grows slowly thereafter, but rarely occludes the pulp chamber. Fig. 1 diagramatically illustrates the details of the development. When the roots are nearing completion, or have just been completed (Fig. 1, *a*), a cap of clear irregular secondary dentin is laid down on the floor of the pulp chamber. Following this, secondary dentin is deposited along the root canals (Fig. 1, *b*) and on the roof of the pulp chamber. As the teeth become older, there is a further thickening of the physiological secondary dentin (Fig. 1, *c*).

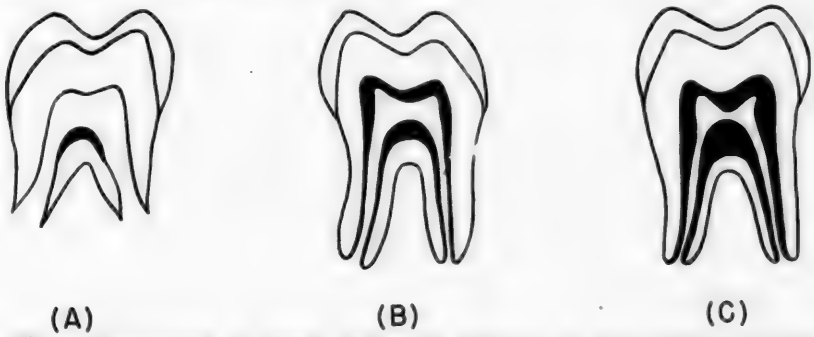

Fig. 1.—Development of typical multirooted molars: (*a*) root formation incomplete; (*b*) root formation recently completed; (*c*) mature tooth. The dark areas indicate secondary physiological dentin.

Fig. 2 is a graphic representation of the correlation between the degree of development of secondary physiological dentin and the chronological age of the tooth. Although the various erupted molars represented in this figure are known to vary somewhat in regard to the completion of root formation, examination of Fig. 2 shows that the trend in development is quite similar for the entire group examined. The graphic presentation of these data also indicates the fact that this tissue develops after root formation is complete and continues to be laid down for some time, and then growth becomes almost if not completely retarded. In comparing the state of development in erupted with nonerupted molars, also shown in Fig. 2, it is evident that the trend in development

of the nonerupted teeth is, in all respects, quite similar in the two groups represented, and further that physiological secondary dentin develops to maturity whether the tooth erupts or does not erupt.

Single-Rooted Teeth.—Examination of a group of forty-nine erupted and eighteen unerupted single-rooted teeth ranging from 7 to 65 years of age reveals a trend in development similar to that described for multirooted teeth.

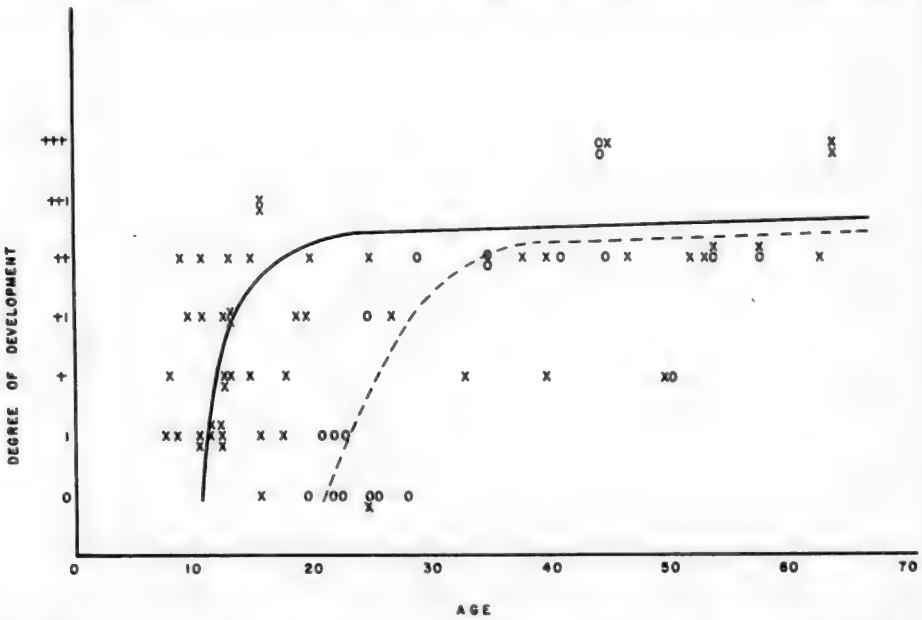

Fig. 2.—Analysis of development of physiological secondary dentin in relation to age of erupted and unerupted molars. (1) The fifty-one erupted multirooted teeth are indicated by *X* and a solid line. (2) The twenty impacted third molars are symbolized by *O* and a broken line. (3) The degree of development is depicted as follows: (a) physiological secondary dentin completely absent, 0; (b) physiological secondary dentin present on the floor of the pulp chamber, +; (c) physiological secondary dentin appearing on the floor and roof of the pulp chamber and a thin lining on the roots, ++; (d) thickening of the physiological secondary dentin circumpulpally, +++.

The physiological secondary dentin appears first in the coronal region of the root (Fig. 3, *a*) and as the tooth matures, a new layer develops apically; finally, the root canal becomes completely lined with it (Fig. 3, *b*). Observations on the single-rooted teeth are represented graphically in Fig. 4. As shown in this figure, the physiological secondary dentin in this group also matures a few years after root formation is complete; following this, growth is inhibited. Both erupted and unerupted teeth disclose a similar development in regard to physiological secondary dentin.

MORPHOLOGY OF PHYSIOLOGICAL SECONDARY DENTIN

Multirooted Teeth.—Fig. 5, *a* is a photograph of an erupted lower second molar of a patient 54 years of age. Note the dense regular physiological secondary dentin lining the roof of the pulp chamber and the clear irregular dentin lining the floor of the pulp chamber. The roots of this specimen show regular

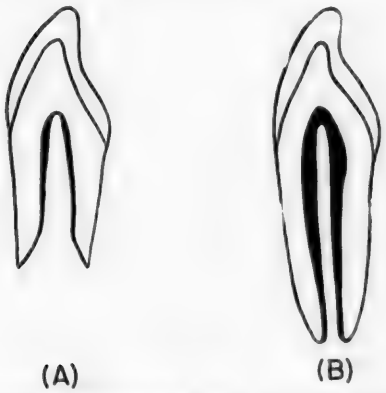

Fig. 3.—Development of typical single-rooted teeth: (a) root formation nearing completion; (b) mature tooth. The dark areas indicate secondary physiological dentin.

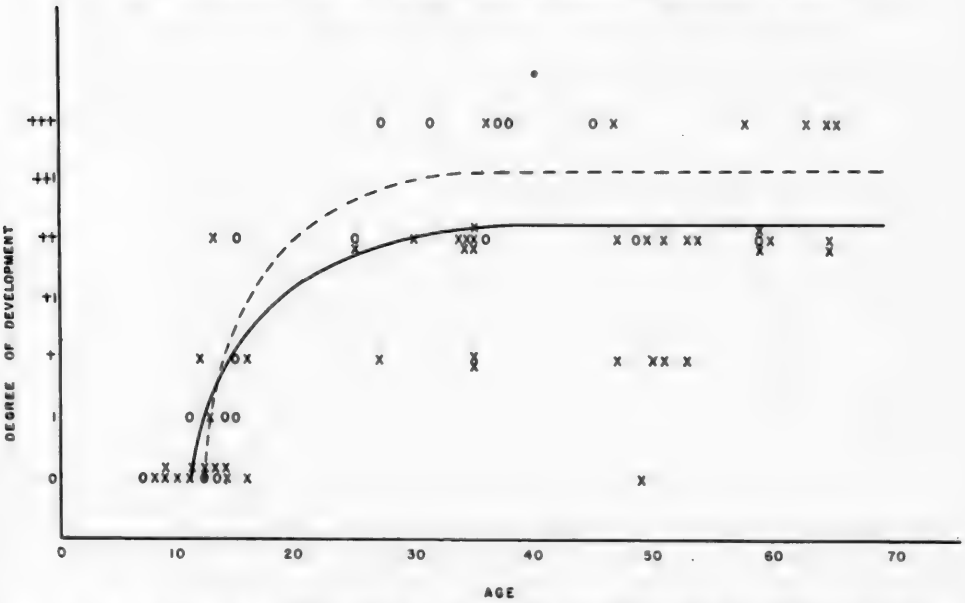

Fig. 4.—Analysis of development of physiological secondary dentin in relation to age of forty-nine erupted and eighteen unerupted single-rooted teeth. Symbols are identical with those used in Fig. 2.

physiological secondary dentin in the region of the crown; approaching the apex, the tubules become progressively more irregular and fewer in number. Fig. 5, *b* is of interest since it is a photograph of a completely unerupted third molar obtained from the same patient as the erupted tooth shown in Fig. 5, *a*. Of particular interest is the fact that physiological secondary dentin in the unerupted tooth is in all respects identical with that shown in the erupted tooth Fig. 5, *a*.

Single-Rooted Teeth.—Erupted and unerupted single-rooted teeth also present similar morphology of secondary dentin. The unerupted canine in Fig.

6 represents a situation typical for both groups. Dense regular physiological secondary dentin is observed near the crown, and as the apex is approached, the dentin becomes more irregular and less dense. At the extreme apex, one often sees a clear matrix containing a few irregular tubules.

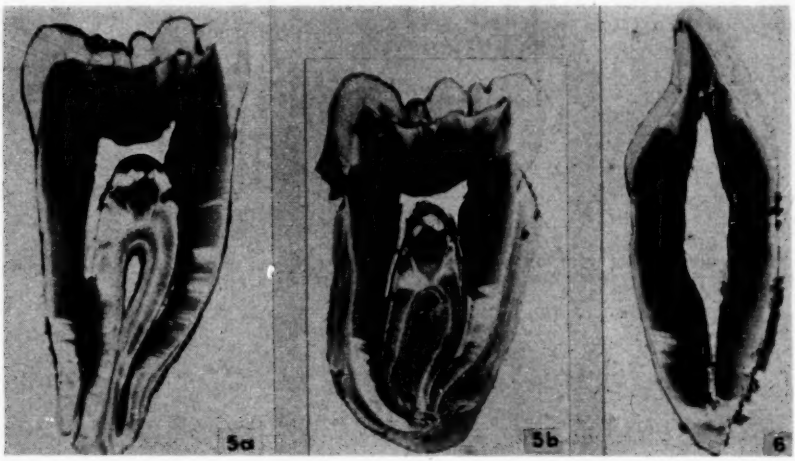

Fig. 5.—*a*, Mature, normal, erupted second molar of patient 54 years of age. *b*, Unerupted third molar of the same patient.
Fig. 6.—Unerupted canine of 31-year-old patient.

Deciduous Teeth.—Sections of noncarious, overretained, deciduous teeth also reveal the presence of physiological secondary dentin. Examination of a typical section of one of these specimens (Fig. 7) not only shows a sharp line of demarcation between primary and adventitious secondary dentin, but also a difference in consistency of this tissue in the coronal and apical regions.

EXTRAORAL TEETH

In order to test the concept that physiological secondary dentin is related to function, a number of published photographs of teeth obtained from ovarian dermoid cysts were examined. They reveal the fact that physiological secondary dentin lines the pulp chamber in a fashion similar to that observed in erupted teeth. A tooth from an ovarian dermoid cyst was sectioned by the author (Fig. 8, *a*). As shown in Figs. 8, *a* and 8, *b*, all the elements of a normal tooth are present: enamel, cementum, pulp chamber, primary dentin, and physiological secondary dentin. A higher magnification of the root (Fig. 8, *b*) clearly illustrates the line of demarcation between the primary dentin and physiological secondary dentin.

DISCUSSION

This study is a continuation of a previous study[1] which dealt with the morphology and incidence of circumpulpal and adventitious secondary dentin in human teeth. Previous reports which deal with this topic are conflicting in many respects. Reich,[2, 3] for example, maintained that circumpulpal secondary dentin is the result of functional stimuli. In an attempt to test this

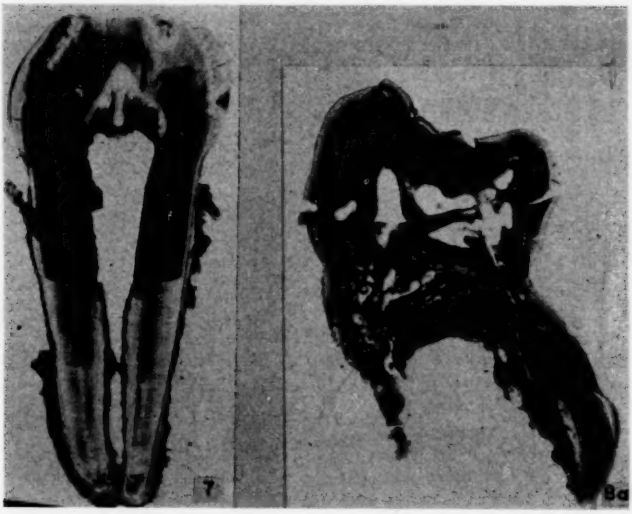

Fig. 7.—Deciduous canine of 14-year-old patient.
Fig. 8.—*a*, Section of a tooth from an ovarian dermoid cyst.

Fig. 8.—*b*, High-power view of the root of the tooth shown in Fig. 8, *a*.

hypothesis, specimens were selected to yield data which would clarify this contention. In this study, normal mature permanent teeth were examined and compared with deciduous and unerupted teeth and with teeth obtained from ovarian cysts. The data and the specimens show that the circumpulpal dentin occurs in nearly every mature tooth examined, regardless of whether the tooth had been in functional occlusion, unerupted or located extraorally. It thus seems obvious that circumpulpal secondary dentin is a regular part of normal growth of the tooth and not the result of "functional" stimuli, as Reich suggested.

This study also shows that the formation of physiological secondary dentin is not a rapid process which continues throughout the life of the individual. After a certain thickness is laid down, the rate of growth is considerably reduced; the metabolism of the tissue is maintained by the pulpal tissues which, under certain conditions, can still deposit adventitious secondary dentin in response to injurious stimuli. Rarely does physiological secondary dentin completely occlude the root canal. When the root canal is occluded, it is usually by adventitious secondary dentin or pulp stones, both of which are usually the result of pathological conditions.

The morphological varieties of physiological secondary dentin are similar to those of adventitious secondary dentin and consist of all the gradations from the dense regular arrangement of the dentinal tubules to clear dentin.

Kronfeld[7] postulated that irregular secondary dentin is the result of an atrophic pulp and degenerated odontoblasts in the aged tooth. Irregular dentin is found in teeth long before the teeth reach maturity or old age; thus, it is difficult to explain the different morphological types of secondary dentin that are deposited in the same tooth on this basis. Differences in the physiology or metabolism of the dentin-producing tissues still awaits further study.

CONCLUSIONS

1. Physiological secondary dentin is a definite topographical layer of dentin which is deposited circumpulpally and follows a definite growth pattern.
2. Physiological secondary dentin is a tissue which is present whether the tooth is functional or nonfunctional, oral or extraoral, and should thus be differentiated from primary dentin and adventitious secondary dentin. There is no evidenc that this tissue is dependent in its formation on functional stimuli.
3. There are several morphological varieties of physiological secondary dentin, ranging from a dense regular dentin to a clear irregular dentin.
4. Deciduous teeth appear to have a development and morphology of the physiological secondary dentin similar to that of permanent teeth.

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